Ecology of diseases transmitted by mosquitoes to wildlife

edited by:

Rafael Gutiérrez-López, James G. Logan
and Josué Martínez-de la Puente

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of vector-borne diseases
Volume 7

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Ecology and control of vector-borne diseases

The importance of vector control

Vector borne diseases account for 17% of all infectious diseases worldwide, causing 700,000 deaths annually. Although we have made significant progress towards understanding vector biology and ecology, vector control is facing many significant challenges. Current control almost entirely relies on insecticides and insecticide-treated bed nets, but many vector species have now developed resistance to insecticides and there is a significant dearth of alternative compounds. As a result of climate change, vectors are expanding their range and we face an ever-increasing and unpredictable threat of outbreaks with possible outcomes we don't fully understand. Malaria control is at a standstill. There are almost 100 million cases of dengue each year, with more than 3.9 billion people in more than 128 countries at risk. The Zika virus epidemic in 2015, was a wakeup call.

It is time for a revolution in vector control. We need to heighten our understanding of vector biology and ecology and we need a new generation of innovative and novel technologies for vector control that can be implemented quickly. This will include challenging the status quo, pushing boundaries and evaluating and implementing new tools more efficiently.

What we can do

We are living in an exciting point in history. Science has advanced such that we can not only think beyond conventional control methods, new and exciting technologies are on the horizon and have the capacity to transform the vector control landscape. Wiping out vector borne diseases could be a reality in our lifetime.

As scientists continue to innovate and develop better methods in molecular biology, we are beginning to unravel elements of vector biology and ecology that allow the development of potential game changing tools such as gene drive, including CRISPR and Wolbachia. As technology becomes smaller, smarter and more affordable, we are facing a future where the sort of technologies you might have only imagined could be possible in sci-fi movies, is now becoming a reality. Drones are being developed that seek out breeding sites, solar powered traps are being developed with automated vector identification technologies using machine learning. Although there are significant hurdles to overcome, we have the capacity to collect data on a scale never seen before and model it for evidence-based predictions to respond to disease outbreaks. It is probably one of the most exciting times for vector researchers with opportunities to be profoundly impactful.

How the book series helps

This series of books aims not only to educate and showcase the latest advances in understanding vector ecology and vector control, but to inspire, promote and stimulate new and innovate ideas.

Our past topics have already explored complex and important issues like ticks and Lyme disease, olfaction and emerging vector borne diseases in Europe. Going forward, the series will explore state-of-the-art thinking and science, including game changing technologies and interventions, based on molecular biology and genetics, digital technology and artificial intelligence, study design for efficient and robust evaluation of control tools, social science and the need for multisectoral collaboration. The series will also be tackling some of the biggest issues, including the environment and minimising the use of toxic insecticides, and exploring how climate change and the concept of planetary health, will impact on vector ecology and control.

James G. Logan is the senior editor of the series. Each volume is co-edited by one or more guest editors, which in Volume 7 are Rafael Gutiérrez-López and Josué Martínez-de la Puente. The editors of the current volume are well-known experts in the field of ecology, control and management of vector borne diseases.

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Introduction

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1. Introducing the role of mosquitoes in the transmission of pathogens to wildlife

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Abstract

Mosquitoes play a central role in the transmission of pathogens causing important diseases to humans and other animals. The incidence of zoonotic diseases has increased in recent decades, many of them caused by pathogens transmitted by mosquitoes. Due to the relevance of these diseases in public and animal health, medical and veterinary entomologists have traditionally focused their studies on the impact of mosquitoes, among other vectors, in diseases such as malaria, West Nile fever or dengue. However, the relevance of mosquitoes in the transmission of pathogens affecting wildlife have been comparatively neglected. The current volume of *Ecology and Control of Vector-Borne Diseases* series highlights significant and novel aspects of the ecology of diseases transmitted by mosquitoes to wildlife, contributing to the better understanding of their epidemiology. We hope this volume will influence to improve our understanding of the dynamics of transmission of mosquito-borne diseases in the wild and provide updated information on the surveillance, control and epidemiology of mosquito-borne zoonotic diseases.

Keywords: arbovirus, One Health perspective, parasites, vector-borne diseases

The incidence of emerging infectious diseases (EIDs) has increased in the past three decades. Nearly 75% of these EIDs have a zoonotic origin, with pathogens naturally circulating in animal reservoirs and, accidentally, infecting humans (Taylor *et al.* 2001). Many of these zoonotic diseases are transmitted by insect-vectors, with mosquitoes playing a central role in their transmission (Pereira-dos Santos *et al.* 2020). Different factors affected by global change have been highlighted as relevant to explain the increase in the incidence of EIDs. The improved efficiency of transportation of materials and people worldwide, together with the introduction of invasive species and processes affected by the habitat anthropization, such as the land use change by deforestation and urbanisation, are driving the emergence of zoonotic diseases, most of them strongly affecting the life cycle, distribution and populations of mosquitoes involved in their transmission (Ferraguti *et al.* 2016).

Globalisation has favoured changes in the natural distribution of pathogens and animals, including mosquitoes, where their introduction or reintroduction may represent a risk to human and environmental health. Animals may carry pathogens and introduce them into new ecosystems with consequent deleterious effects on wildlife/domestic populations and changing the ecological

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balance in an area. In the case of mosquitoes, their introduction in new areas could produce human disturbances due to their bites, but more importantly, may affect the epidemiological scenario of different pathogens allowing their transmission in areas where competent vectors were previously absent. One of the main examples of that is the introduction of the avian malaria parasite *Plasmodium relictum* and their mosquito vectors in Hawaiian Islands, allowing their circulation in the area which highly contributed to the decline of native bird species (Atkinson *et al.* 2010). Imported materials such as tires and/or bamboo, have been recognised as a major factor determining the global spread of *Aedes albopictus* mosquitoes where it plays a key role in the transmission of pathogens in introduced areas. In Europe, *Ae. albopictus* contributed to the transmission of locally circulating parasites, such as *Dirofilaria immitis* in Italy (Cancrini *et al.* 2003), and imported pathogens affecting humans including Dengue virus (Aranda *et al.* 2018), Zika virus (Brady and Hay 2019) and Chikungunya virus (Watson 2007).

One of the main factors affecting the transmission dynamics of pathogens by mosquitoes is their blood feeding patterns (Takken and Verhulst 2013). The blood feeding habits of mosquitoes have been known since, at least, the Middle Eocene, as supported by the use of non-destructive mass-spectrometry analysis to identify the host´s oxygen-carrying group of haemoglobin present in the abdomen of fossil mosquitoes (Greenwalt *et al.* 2013). Nowadays, the use of molecular techniques applied to the study of recently engorged females have revealed the ability of mosquitoes to feed on blood from a diversity of animals, from ectotherms including amphibians and fishes to birds and mammals, including humans. For example, *Ae. albopictus* females feed on blood from fish, birds and mammals with, at least, 20 mammal and 5 bird host species identified in their blood meals (Cebrián-Camisón *et al.* 2020). Consequently, mosquitoes are able to transmit pathogens to different taxa. For instance, in addition to the previously mentioned avian malaria parasites, birds are common hosts of mosquito-borne pathogens including nematodes and viruses, some of them considered zoonotic. Amphibians and reptiles also harbour a wide variety of vector-borne pathogens with mosquitoes playing a key role as vectors involved in their transmission (Matta *et al*. 2022; Chapter 3 of this book).

The ability of mosquitoes to feed on different vertebrate species further supports their role in the ability of transmission of pathogens between them, affecting the epidemiology of different diseases. Zoos and wildlife parks are excellent scenarios to study the patterns of parasite transmission, as they host animals maintained in captivity, native wild animals and human visitors. In addition, they provide suitable environments for the breeding and maintenance of mosquito populations potentially favouring the transmission of mosquito-borne pathogens (Heym *et al.* 2019). Penguins are common hosts of mosquitoes, including those carrying avian *Plasmodium* parasites (Martínez-de la Puente *et al.* 2020) which produce deleterious effects on these animals supporting the necessity to develop treatment protocols (Grilo *et al.* 2016).

Altogether, these examples represent the necessity to improve our knowledge on the ecology of mosquito-borne diseases that affect wildlife which is the main aim of the chapters included in this seventh volume of *Ecology and control of vector-borne diseases*. This volume uses a multidisciplinary approach to provide a general overview of the importance of diseases affecting wildlife from different disciplines from conservation biology to public health, considering the *One Health* paradigm.

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The main wildlife pathogens transmitted by mosquitoes

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2. Mammal´s pathogens transmitted by mosquitoes

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Abstract

Mosquito-borne pathogens are an important challenge for public and animal health. In the last years, invasive mosquito species have spread globally, resulting on emerging diseases in many regions of the world. A combination of several factors, such climate change, globalisation and transport have been associated to this phenomenon and scientific predictions indicate that this pattern will continue during the next decades. It is not yet clear to which extent the introductions of these vectors will negatively affect wildlife ecosystems. Most information on mosquito-borne diseases is often limited to those of medical and/or veterinary relevance, while our understanding of pathogens affecting wildlife is limited. The detection of mosquito-borne diseases in wild mammals is essential in their conservation, but also because it helps detecting emerging mosquito-borne diseases in humans. Furthermore, preventing the spillover of wildlife pathogens to humans requires a deep understanding of the dynamics of the disease in the reservoir hosts and this information is still scarce. We present a comprehensive review of the mosquito-borne pathogens that affect wild mammal species, particularly viruses and parasites. In relation to the latter, many have been described morphologically prior to the introduction of molecular techniques and therefore such information must be critical assessed. Therefore, the number of pathogens included in this chapter is far from complete but includes those with most stable information.

Keywords: bats, rodents, parasites, viruses, mosquito-borne, ungulates pinnipeds

Bats and their role as reservoirs of mosquito-borne diseases

Bats are one of the most diverse groups of mammals in the planet, only second to rodents (Burgin *et al.* 2018), and the only one with the capacity of flight (Omatsu *et al.* 2007). These animals have received wide attention in recent years as these have been involved in the majority of the recent emerging zoonosis worldwide (Brierley *et al.* 2016). Although, they provide unquestionable critical services to ecosystems, such as pollination and seed dispersal, these positive aspects have been eclipsed by their association with virulent zoonotic pathogens. This has resulted in a negative public perception of the group which may, at the same time, negatively impact the conservation efforts (Rocha *et al.* 2020). Taking into consideration that bats actively pray on harmful insect pests, including mosquitoes (Puig-Montserrat *et al.* 2020), it would not be unlikely that this situation may result in an increase of transmission of arthropod-borne diseases. There are, however, many gaps in our knowledge on the ecology, biology and history traits of the different taxa within Chiroptera, that impair our understanding of their role in disease transmission (Letko *et al.* 2020).

Viruses

Bats are hosts of more than 80 virus species (Melaun *et al.* 2014). This diversity may be reflective of the species richness within Chiroptera (Mollentze and Streicker 2020). However, there are other

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explanations to the high diversity of viruses found within this mammalian order. Bat species are often observed living in sympatry and this may be related to the diverse assemblage of bats species within the same roost (Luis *et al.* 2013). Bats have long lifespans relative to other animals their size, which may enable viral persistence (Luis *et al.* 2013). However, possibly the most interesting aspect of the bat physiology is their unique immune system (Banerjee *et al.* 2020), which allow them to remain asymptomatic to viral infections, despite persistent viremia and viral replication in multiple organs (Van den Hurk *et al.* 2009). All these peculiarities may explain why Chiroptera are thought to represent a higher risk of transmission of zoonotic viruses than other mammalian orders (Thompson *et al.* 2015).

Bats harbour other mosquito-borne pathogens including parasites (Melaun *et al.* 2014). However, the large majority of the research studies have focused on their role as hosts of the zoonotic viruses. For most known bat-borne viruses, the transmission dynamics does not include insect vectors (Gentles *et al.* 2020). Several bat species are suspected to be reservoir hosts of arboviruses. However, incriminating bat species as reservoir hosts is not an easy task, as it requires experimental infections and not just the mere detection of nucleic acids or antibodies (Fagre and Kading 2019). The family Flaviviridae includes some of the most important emerging viruses in humans and other animals in the world, as their infection has been associated to a high morbidity and mortality. These include Yellow fever virus (YFV), Dengue fever virus (DENV), Zika virus (ZIKV) and West Nile (WNV). The first three are *Aedes*-borne viruses, while WNV is primarily transmitted by *Culex* mosquitoes. The evidence obtained from experimental infections in captive bats indicates that DENV does not replicate and circulate in sufficient levels in these mammals to allow infection in mosquitoes (Aguilar-Setien *et al.* 2008). Furthermore, bats appear to be dead-end hosts of this DENV (Vicente-Santos *et al.* 2017). Similarly, previous attempts to artificially infect bats with YFV, conducted several years ago, have been unsuccessful (Fagre and Kading 2019). However, high levels of neutralising antibodies titer against YFV have been reported in *Rousettus aegyptiacus,* the Egyptian rousette bat in Uganda (Kading *et al.* 2018), indicating that new attempts to infect bats may be needed to clarify their role as Yellow fever reservoirs. The *Culex*-borne Japanese encephalitis virus could be, arguably, considered the most important Flaviviridae member as this causes the most severe clinical manifestations in humans (Gould *et al.* 2017). Bats are likely to be reservoirs of the disease (Solomon 2003). Usutu virus (USUV) is a mosquito-borne Flavivirus included within the Japanese Encephalitis serocomplex and mostly transmitted by *Culex* spp. mosquitoes. Usutu virus disease was possibly introduced in Europe in the 1990s. However, it received little attention until year later it was associated to an outbreak of birds in Vienna (Weissenbock *et al.* 2002). The life cycle naturally include birds and mosquitoes, however, in some parts of Europe DNA from USUV has also been detected in bats that were found dead in southwest Germany (Cadar *et al.* 2014) and later was detected in bats from Belgium.

Chikungunya virus (CHIKV) belongs to the Togaviridae family. Although the enzootic transmission of this virus includes mosquitoes and non-human primates, other vertebrates including bats may be involved in the sylvatic transmission. Stone *et al.* conducted a serological surveillance of several arboviruses in West Indies and found a 36% prevalence of antibodies to the Chikungunya virus CHIKV in Grenada fruit bats (genus *Artibeus* sp.) living in close proximity (<1000 m) to human settlements (Stone *et al.* 2018). Shah and Daniel (1966) artificially infected Indian flying foxes with CHIKV. This resulted in a subclinical infection in animals, showing enough blood viremia to infect mosquitoes (Fagre and Kading 2019). Bats may also be hosts of other members of this virus family, such as the Venezuelan equine encephalitis virus (VEEV). One of the earliest reports of this virus in bats date from the 1970s, when a vampire bat *Desmodus rotundus* was found infected with an epidemic strain of this virus (Guzman-Teran *et al.* 2020). Thompson *et al.* (2015) detected

VEEV-specific antibodies in bats from Trinidad and more recently, Sotomayor-Bonilla *et al*. (2018) detected this virus in three bat species in Mexico. The role of bats as reservoir hosts of the the Madariaga virus (MADV), formerly known as the Eastern equine encephalitis virus appears to be less important (Thompson *et al.* 2015). Although many aspects of this virus transmission remain unknown (Vittor *et al.* 2016). Further studies, including artificial infections are required to clarify the status of bats as hosts of this virus species.

The Bunyaviridae family comprises more than 300 virus species that cause viral haemorrhagic and arboviral fevers in a wide variety of hosts including arthropods, mammals (Soldan and Gonzalez-Scarano 2005) and even plants (Van Vuren *et al.* 2017). These viruses are transmitted by arthropods and rodents, infecting humans, occasionally. Within this family, the Rift Valley Fever virus (*Phlebovirus* genus) is transmitted by mosquitoes of the genera *Aedes* and *Culex*, although it may also be transmitted by sandflies (Pepin *et al.* 2010). Several outbreaks of the disease have been associated with a dramatic increase in mosquito densities after periods of rainfall (Bicout and Sabatier 2004). Although the reservoirs of the disease have not been clarified yet, bats have been suggested as potential reservoir species (Boiro *et al.* 1987). Disease transmission may not only occur throughout biting, but also by the ingestion of infected mosquitoes (Oelofsen and Van der Ryst 1999). The Guamá (GMAV) and Catú viruses (CATUV) belong to the *Orthobunyavirus* genus and have public health importance since both can cause acute febrile syndrome in humans (Melaun *et al.* 2014). These are transmitted by *Culex* mosquitoes and have been isolated from bats (Newman *et al.* 2011). Other mosquito-borne viruses from this family have artificially infected cell lines derived from bats and mosquitoes, including the closely related Bwamba (BWAV), Pongola (PGAV) and Nyando (NDV) viruses (Groseth *et al.* 2014). Although the role of these animal hosts must be fully dilucidated.

Parasites

The number of studies conducted on bat haemosporidians is lower in comparison to those studying virus and possibly neglected (Megali *et al.* 2011). Dipteran species, other than mosquitoes are reported vectors of these parasites. For instance, parasites of the genera *Hepatocystis* and *Polychromophilus* are important haemoparasites of bats. Despite their close phylogenetic relationship to *Plasmodium* parasites, these are transmitted by *Culicoides* species (Garnham *et al.* 1961) and bat flies (Ramasindrazana *et al.* 2018). A comprehensive review of the investigations conducted in haemosporidian parasites was conducted by Melaun et al (2014). Authors reported haemosporidian infections are common in Chiroptera. However, despite bats are bitten by mosquito species that are vectors of human diseases, their role in the zoonotic transmission is still unknown.

Mosquito-borne pathogens in rodents and other small mammals

Rodents (order Rodentia) are the largest group of extant mammals. With more than 2,270 extant species more than 10% of are zoonotic hosts of pathogens (Han *et al.* 2016). Rodents are considered a serious concern as they serve as reservoirs of emerging viruses (Luis *et al.* 2013). Rodents are responsible for the transmission of more than 35 human pathogens worldwide (CDC 2010) and are reservoirs of more than 66 zoonotic pathogens, including viruses, bacteria, fungi, protozoa and helminths (Han *et al.* 2015). They share with bats some characteristics that enhance their role as reservoir of human pathogens: high species richness, synanthropic behaviour and hibernation capacity (Luis *et al.* 2013). Furthermore, they also possess a high reproductive potential, forming large and highly dense groups, which increases the likelihood of pathogen spillover to humans

(Catenacci *et al.* 2018). Not only rodents play an important role in the transmission but also in the amplification of pathogens (Springer *et al.* 2016), implying a rapid viral multiplication that facilitate transmission.

Viruses

Some rodent-borne diseases are transmitted by vectors, making more than 30% in some areas of the world (Rabiee *et al.* 2018). The large majority of such reports are associated to ticks, fleas or mites vector species (CDC 2010), while there are fewer reports of mosquitoes. We present here some of these reports. Dengue virus (DENV) is mainly transmitted between humans and mosquitoes. Neutralising antibodies of DENV have been detected in small mammals (De Thoisy *et al.* 2009). However, further studies are required to elucidate their role in the transmission of DENV in these mammals. The Western Equine encephalomyelitis virus (WEEV) belongs to the Togaviridae family of viruses. Although WEEV mainly circulates in birds and mosquitoes, it causes disease in humans and equids occasionally (Bergren *et al.* 2014). In South America, it is likely that a mosquito-rodent cycle occurs, including rice rats, rabbits and hares (Pfeffer and Dobler 2010). A significant decrease of WEEV human infections and virus detection in mosquito pools has been reported in North America (Bergren *et al.* 2014), indicating that this pathogen may be decreasing in prevalence. Also caused by members of this group, the Mayaro virus disease (MAYVD) is an emerging zoonosis transmitted by mosquitoes from the *Haemogogus* genus, with non-human primates as their primary hosts (Esposito and Da Fonseca 2017). Rodents have also been postulated as hosts of this virus in the Amazon area (Ali *et al.* 2019) although their role in the transmission remains to be understood (De Thoisy *et al.* 2003). Other viruses from this family such as the Venezuelan Equine Encephalitis Virus and the Chikungunya virus are thought to have rodents as hosts in sylvatic cycles. Spiny rats (*Proechimys* sp.) have resulted asymptomatic to the infection and are thought to be the main reservoirs of VEEV in the Americas (Carrara *et al.* 2005). On the other hand, the results from experimental infection of small mammals with the CHIKV indicates that rodents may not be efficient amplifier hosts (Bosco-Lauth *et al.* 2016).

Some of the first reports of West Nile Virus (Flaviviridae) infecting mammals have been conducted in rodents. This virus has been reported in squirrel species from the genus *Sciurus* spp., the eastern chipmunk *Tamias striatus* and eastern cotton tail rabbit *Sylvilagus floridanus*. These species develop a high enough viremia to infect mosquitoes (Root 2013). Usutu virus is a *Culex*-associated member of this family. This virus has been isolated and sequenced from five small mammals in Senegal, two of which are rodents: the black rat, *Rattus rattus* and the multimammate rat, *Mastomys natalensis*, in addition to a shrew species from the *Crocidura* genus. The clinical examination determined these animals were asymptomatic to the infection (Diagne *et al.* 2019).

La Crosse virus (LACV) belongs to the Bunyaviridae family, within the California encephalitis virus serogroup. Mosquitoes of the genus *Aedes* as their primary vectors (Harding *et al.* 2019). This virus can cause encephalitis in humans and other animals (Harding *et al.* 2019). Artificial infections of chipmunks have demonstrated their role as amplifier hosts, while intrauterine infection of Mongolian gerbils have shown proved it can be vertically transmitted (Osorio *et al.* 1996). The Tahyna virus (TAHV) is another member of the California serogroup. This mosquito-borne pathogen is transmitted by several species of mosquitoes while hares, rabbits and hedgehogs act as their amplifying hosts (Bennett *et al.* 2011, Soldan and Gonzalez-Scarano 2005).

Parasites

In relation to mosquito-borne parasites in rodents, those from the *Plasmodium* genus have been well-characterised as they serve as models to understand the biology of malaria infection (De Niz and Heussler 2018). However, most of the studies are restricted to their role of rodents as biological models with limited information on the disease these parasites cause in wild rodent populations. One of the few surveillance reports was conducted by Boundenga *et al.* (2019). These authors studied haemoparasites present in rodents from Gabon (Central Africa) and found species *Plasmodium vinckei* and *Plasmodium yoelii* infecting five rodent species in Gabon, Central Africa. Lutz *et al*. (2016) reported no malaria parasites in 286 rodents and shrews from East Africa and found no infections. According to the authors these results may be related to non-specificity of the primers used for DNA amplification.

Mosquito-borne pathogens of sea mammals

There are few studies reporting mosquito-borne diseases in sea mammals, however some outbreaks have been reported in pinnipeds, a group that includes walruses, sea lions and fur seals. Pinnipeds sometimes live near humans and spend large periods of time resting in terrestrial ecosystems. These mammals are then more exposed to mosquito bites than other marine fauna and therefore, more susceptible to mosquito-borne pathogen infections (Alho *et al.* 2017; Figure 1).

Viruses

The incidence of mosquito-borne viruses in sea mammals is not well understood and it is likely to be underestimated. There is limited evidence of the interaction of mosquito vectors and sea

Figure 1. Exposure to mosquito-bites and to mosquito-borne pathogens is thought to be higher in captive sea mammals as a result of increased resting behaviours and close proximity to coastal ecosystems where mosquitoes are more abundant.

mammals (Reeves and Gillett-Kaufman 2020). However, some reports indicate that such interaction occurs, at least in captive animals. The first of such a reports occurred in a captive killer whale *Orcinus orca*, which died from St Louis encephalitis virus in Orlando, Florida (Buck *et al.* 1993). More recently, in a marine park in Texas, Leger *et al.* (2011), reported a fatal West Nile virus infection in an adult whale. However these cases were reported in captive animals, and its susceptibility seem to be the result of their logging behaviour, which consists on floating at surfaces, which turns them more susceptible to mosquito bites (Jett 2012). The only report of a mosquito-borne virus infection in a wild sea mammals occurred in dolphins (*Tursiops truncates*) in the Indian River Lagoon in Florida (Schaefer *et al.* 2009). Some cases of West Nile Virus disease have also been reported in pinnipeds. Similar to the infection reported in humans, that one in harbour seals was characterised by signs of neurological disfunction, including tremors and twitching, breathing difficulties and gastrointestinal compromise (Del Piero *et al.* 2006).

Parasites

The mosquito-borne nematode species *Dirofilaria immitis* (Spiruruda: Onchocercidae) shows a moderate vertebrate host specificity. This has been reported in several species of captive pinnipeds. The first reports in pinnipeds date back to the 1970s in North America, when the parasite was reported in the harbour seal, *Phoca vitulina* (Medway and Wieland 1975) and in the California sea lion *Zalophus californianus* in zoological parks of Florida and Louisiana (Forrester *et al.* 1973, White 1975). In Europe, Alho *et al.* (2017) recently reported a *D. immitis* infection in the South African fur seal, *Arctocephalus pusillus pusillus* housed at an oceanographic park in the Algarve region, in Portugal.

These studies have been conducted in captive pinnipeds, leaving many open questions regarding the epidemiological role of filarioid nematodes in nature. The first study conducted in wild pinnipeds has been recently reported by Farriols *et al.* (2020), who conducted a parasitological surveillance of filaroid nematodes in free-ranging California sea lions in Mexico. Interestingly, despite the high parasitic load, infected animals appeared healthy, indicating that California sea lions may be tolerant to filaroid infections. Clinical manifestations of the disease may vary depending in the location of the parasites. Asymptomatic infections have been reported particularly when the parasites remain located in the heart and associated blood vessels while in some cases severe clinical signs have been observed, including cardiopulmonary impairment, coughing and breathing difficulties (Forrester *et al.* 1973, Medway and Wieland 1975), pulmonary emphysema and haemorrhage (Alho *et al.* 2017).

Mosquito-borne viruses in ungulates

Ungulates including Artiodactyla (even toed ungulates) and Perissodactyla (odd-toed ungulates) are widely spread in different ecosystems of the planet from the tropical forest to the arctic tundra. These mammals are key elements of terrestrial ecosystems, with the capacity of altering ecosystems structure and composition (Velamazan *et al.* 2020). With more than 400 species recorded, their abundance, distribution and extensive knowledge of their ecology made them good models of disease ecology (Jolles and Ezenwa 2015). The role of ungulates as hosts of zoonotic diseases is well established, with more than 250 human pathogens currently described (Woolhouse and Gowtage-Sequeria 2005). Their parasites are well characterised (Stephens *et al.* 2019) and recently an increasing number of viral diseases have been reported in these mammals, including mosquito-transmitted viruses.

Viruses

As it has been described before, the transmission of West Nile virus naturally occurs in bird hosts and mosquito vectors (Jain *et al.* 2020). However, more than 100 mammal species have also been found exposed to the infection of this emerging pathogen, including free ranging and captive animals (Root 2013). Ungulates, such as the Eurasian wild boar *Sus scrofa* and the red deer *Cervus elaphus,* have proved effective biosentinels of West Nile virus (Garcia-Bocanegra *et al.* 2016). Although it is unlikely that they would be amplifying hosts, storage of blood samples may be useful in retrospective monitoring studies of virus circulation (Boadella *et al.* 2012). Another ungulate species considered a good sentinel for the early detection of WNV are white-tailed deer (Palermo *et al.* 2020). WNV antibodies have also been reported in dromedary camels, mouflons, African forest buffalos, reindeers and roe deer (Root and Bosco-Lauth 2019).

The Rift Valley fever virus (RVFV) is another emerging mosquito-transmitted pathogen that infects both ungulates and humans (Linthicum *et al.* 2016). Apart from domestic ungulates, the disease has been reported in buffalo and camels. The infection has been associated to abortions and high mortality in young animals (Calkins and Scasta 2020). The *Culex*-borne Japanese encephalitis virus can use horses and pigs as amplification hosts (Gould *et al.* 2017). In North America, moose populations have tested positive for the Equine encephalitis virus during the 2020 hunting season (Lubelczyk *et al.* 2014). Unexplained deaths in feral horses in the United States, resulted in the discovery of the Malpais Spring Virus (MSPV), also infecting deer, coyotes and gemsboks (Clark *et al.* 1988). There is limited information in the literature on this virus, their host species and epidemiology. Currently, MSPV has been assigned to the family Rhabdoviridae (Vasilakis *et al.* 2013) and it has been isolated from pools of mosquito species *Aedes campestris* and *Psorophora signipennis* (Clark *et al.* 1988). Further studies are required to determine which vector species are involved in the transmission.

Parasites

Filaroid nematodes can infect vertebrates from different orders causing severe economic, medical and veterinary consequences. They are considered one of the main biomedical challenges of this century (Hoerauf *et al.* 2011). Their clinical manifestations are often severe, sometimes causing serious disabilities or the death of the host (WHO 2020). In the last decades, reports of zoonotic filariasis have shown an increasing trend, while only those from mammal hosts have been recorded as zoonotic (Orihel and Eberhard 1998).

The genus *Setaria* Viborg 1795 (Nematoda: Filariidae) is formed by 46 members that live in the abdominal cavities of Artiodactyla, Perissodactyla and Hyracoidea. The life cycle include mosquitoes or flies from the *Haematobia* genus (Curlik *et al.* 2019). *Setaria tundra* Isaichikov and Raevskaya, 1928 is a filarioid nematode that infects free ranging ungulates. This parasite has received attention from the scientific community as it is expanding southwards (Olos *et al.* 2019). The life cycle of *S. tundra* remains to be elucidated, however, mosquitoes of the genus *Aedes* are thought to be the principal vectors of this filaroid nematode (Laaksonen *et al.* 2009). This species appears to be highly prevalent in the northern hemisphere, particularly in Finland, where there are several reports of peritonitis outbreaks in reindeers (*Rangifer tarandus tarandus*) (Laaksonen *et al.* 2007). This nematode has also been reported in Poland (Olos *et al.* 2019), Croatia (Curlik *et al.* 2019), Austria (Ubleis *et al.* 2018, Zittra *et al.* 2015), Denmark (Enemark *et al.* 2017), Hungary (Kemenesi *et al.* 2015) Germany (Kronefeld *et al.* 2014). In Southern Europe it has been reported in Spain (Angelone-Alasaad *et al.* 2016). Other species of the same genus have been reported in ungulates. *Setaria yehi*, is present in north America infecting local species of cervids, causing fibrinous peritonitis (Prestwood and Pursglove 1977). *Setaria digitata* and *Setaria marshalli* have been reported in the abdominal cavities of cattle (Tung *et al.* 2003). In horses the infection of *Setaria* spp. has resulted in aberrant migration of parasites to the eye (Shin *et al.* 2017).

Although human haemosporidian species are among the most widely studied parasites, their number is negligible if we consider large number of haemosporidians lineages in vertebrate hosts. The first reference of malaria parasites in ungulates was recorded by sir David Bruce during a blood survey in duiker antelope in Malawi (Bruce *et al.* 1913). Since then, many other *Plasmodium* species have been described in ungulates, such as *Plasmodium bubalis* in buffaloes (*Bubalus bubalis*) (Sheather 1919). A recent study indicated that up to 25% of all white-tailed deer may be infected with *Plasmodium odocoilei* (Guggisberg *et al.* 2018). Since the first discovery of this parasites in ungulate hosts, description of new species has been based on a morphological approach (Templeton *et al.* 2016). While further molecular analysis may be required to confirm species status.

Mosquito-borne diseases in marsupials

Viruses

Among the few mosquito-borne pathogens reported in marsupials, one of the most relevant is the Ross River Virus (Togaviridae). This is a zoonotic Alphavirus responsible for most mosquito-borne human cases in Australia and Papua New Guinea. In addition to fever, clinical manifestations include polyarthralgia and arthritis in affected people (Harley *et al.* 2001). Marsupials appear to be competent reservoirs of the disease, as the prevalence in these mammals is higher than in the placental ones and birds (Stephenson *et al.* 2018).

The West Nile virus has also been detected in marsupials. The infection resulted in Pulmonary Lepidic-Predominant Adenocarcinoma in *Didelphis marsupialis* in Canada (Lamglait and Lair 2019). WNV had already been isolated from this marsupial species before (Bosco-Lauth *et al.* 2014), however the detection of antibodies had previously indicated this species was somehow resistant to a severe manifestation of the disease (Root and Bosco-Lauth 2019). There need for further information to clarify the effects of the virus in this marsupial host. Opossum appear to be resistant to the infection of the Western Equine Encephalitis virus, although the basis behind such resistance is unknown (Barr 1963).

Parasites

There is limited information on the mosquito-borne parasites present in marsupial species. Scientific reviews of infectious diseases in these mammals often do not include mosquito-borne parasites (Barr 1963), possibly because the lack of studies. In a survey of haemoparasites in French Guiana, de Thoisy *et al.* (2000) found that American marsupials of the family Didelphidae were infected to *Hepatozoon* spp. and filaria parasites but none was found infected with *Plasmodium* species. The first two pathogens may be transmitted by mosquitoes but also by other haematophagous insects. There is one report of a fatal *Plasmodium* sp. infection the Leadbeater's possum (*Gymnobelideus leadbeateri*), the first haemoparasite species reported for this marsupial species (Scheelings *et al.* 2016).

Mosquito-borne pathogens in non-human primates

Mosquito-borne parasites in non-human primates are limited to protozoan parasites of the genus *Plasmodium* and various filarioid helminths. While *Plasmodium* parasites in primates are studied extensively, information about filarioid helminths (esp. of non-zoonotic nematodes) is scarce. While using molecular tools some haemoparasites (e.g. mitochondrial DNA of *Plasmodium* spp.) can not only be detected in blood but also (non-invasive) in faeces, detection remains challenging for other haemoparasites like filarioid helminths (Mapua *et al.* 2018). Considering the close phylogenetic relationship with humans, non-human primates (NHP) share with us several viral pathogens. Furthermore, some of these viruses have their origin in such primates (Tongthainan *et al.* 2020). Frequently NHP are show no clinical signs but have the capacity of transmitting the disease to humans (Jones *et al.* 2008). While sylvatic transmission cycles occur as follow NHP-mosquitoes-NHP, humans can become infected when entering forest habitats (e.g. hunting, deforestation) (Valentine *et al.* 2019). The viruses present in primates, including humans are mainly RNA viruses. More than 50% of these are transmitted by arthropods (De Almeida *et al.* 2019, Pedersen *et al.* 2005).

Viruses

Arboviruses in non-human primates are transmitted in urban and sylvatic cycles. In the case of Yellow fever virus (YFV), it is transmitted by *Aedes* mosquitoes in urban environments, canopy mosquitoes (*Haemogogus* spp. and *Sabethes* spp.) are responsible for the sylvatic transmission (Fernandes *et al.* 2020). In South America, YFV have been reported in Howler monkeys (*Alouatta* spp.), which develop severe clinical manifestations, including hepatic failure (Leal *et al.* 2016). YFV has also been isolated from *Callicebus* sp., *Callithrix* sp. and *Sapajus* sp. (Sacchetto *et al.* 2020). Dengue fever has also been reported in urban and forested settings (Simmons *et al.* 2012). Although there are reports on humans acquiring Dengue sylvatic lineages, these has not been associated to large human outbreaks (Cardosa *et al.* 2009). NHP develop Chikungunya virus infection with similar clinical manifestations to those reported in humans (Labadie *et al.* 2010). Macaque monkeys showed neutralising antibodies against CHIKV, DENV and Zika virus in Thailand, indicating the occurring of sylvatic cycles of these viruses (Tongthainan *et al.* 2020). Despite strong evidence of transmission of these viruses in NHP, some species, such as the African Green monkeys, appear refractory to the infection (Valentine *et al.* 2020). Recently, various authors have reported the presence of antibodies against West Nile virus in South American and African primates (summarised by Root and Bosco-Lauth (2019). Other less-known viruses transmitted by mosquitoes have also been recorded in NHP. These are the Spondweni virus, Mayaro virus and the O´nyong-nyong virus (Valentine *et al.* 2019).

Parasites

Non-human primate but also human malaria parasites are protozoa of the genus *Plasmodium* transmitted by anopheline mosquitoes. Overall host-specificity in both, the intermediate primate host, but also in the definitive mosquito host (where sexual reproduction takes place), is given. This means that a certain *Plasmodium* parasite can be transmitted by certain anopheline species only and can only infect one or a limited number of primate host species.

The field on non-human primate malaria is complex. Older literature (e.g. Coatney 1971) is based on morphological specification only. The upcoming of molecular tools lead to the specification of non-human primate malaria parasites like *Plasmodium knowlesi* in humans. In non-human primates 27 *Plasmodium* species were documented, but it is estimated that this number will increase (Martinelli and Culleton 2018). Furthermore, non-human primates were (and are still) used as animal models for human malaria parasites. An overview of non-human malaria parasites is given in Table 1.

Traditionally the human malaria species inventory consists of *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. With the upcoming of molecular tools this number increased to six species. *P. ovale* was demonstrated to be two non-recombining, genetically distinct, sympatric occurring parasites – namely *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland *et al.* 2010). Moreover, *P. knowlesi*, formerly known as a primate malaria parasite only, was included (White 2008).

P. falciparum is the most important human malaria parasite. It is a member of the subgenus *Laverania* which also includes *Plasmodium praefalciparum*, *Plasmodium adleri*, and *Plasmodium blacklocki* from western lowland gorillas, *Plasmodium reichenowi*, *Plasmodium gaboni*, and *Plasmodium*

Table 1. Plasmodium *species in non-human primates (modified after Martinelli and Culleton (2018).*

Parasite	Host	Region
Plasmodium schwetzi	Chimpanzee (*Pan troglodytes*)	Africa
Plasmodium rodhaini	Chimpanzee (*Pan troglodytes*)	Africa
Plasmodium carteri	Chimpanzee (*Pan troglodytes*)	Africa
Plasmodium reichenowi	Chimpanzee (*Pan troglodytes*)	Africa
Plasmodium gaboni	Chimpanzee (*Pan troglodytes*)	Africa
Plasmodium billcollinsi	Chimpanzee (*Pan troglodytes*)	Africa
Plasmodium praefalciparum	Gorillas (*Gorilla gorilla*)	Africa
Plasmodium adleri	Gorillas (*Gorilla gorilla*)	Africa
Plasmodium blacklocki	Gorillas (*Gorilla gorilla*)	Africa
Plasmodium gonderi	Sooty mangabey (*Cercocebus atys*)	Africa
Plasmodium pitheci	Orangutan (*Pongo pygmaeus*)	Asia
Plasmodium silvaticum	Orangutan (*Pongo pygmaeus*)	Asia
Plasmodium eylesi	Lar gibbon (*Hylobates lar*)	Asia
Plasmodium jefferyi	Lar gibbon (*Hylobates lar*)	Asia
Plasmodium hylobati	Gibbon (*Hylobates sp.*)	Asia
Plasmodium youngi	Siamang (*Symphalangus syndactylus*)	Asia
Plasmodium fieldi	Southern pig-tailed macaque (*Macaca nemestrina*)	Asia
Plasmodium simiovale	Toque macaque (*Macaca sinica*)	Asia
Plasmodium coatneyi	Rhesus macaque (*Macaca mulatta*)	Asia
Plasmodium fragile	Bonnet macaque (*Macaca radiata*)	Asia
Plasmodium knowlesi[1]	Long-tailed macaque (*Macaca fascicularis*)	Asia
Plasmodium cynomolgi[1]	Macaques and leaf monkeys	Asia
Plasmodium inui[1]	Macaques and leaf monkeys	Asia
Plasmodium brasilianum[1]	Bald uakari (*Cacajao calvus*)	America
Plasmodium simium[1]	Howler monkeys (*Alouatta sp.*)	America
Plasmodium lemuris	Collared brown lemur (*Eulemur collaris*)	Madagascar
Plasmodium girardi	Lemur (*Lemur sp.*)	Madagascar

[1] Zoonotic (Baird 2009).

billcollinsi from chimpanzees, and *Plasmodium lomamiensis* from bonobos (summarised in Sharp *et al.*(2020)). In the wild *Laverania* species are strictly host specific. *P. falciparum* arose from the gorilla parasite *P. praefalciparum* and is the evolutionary youngest human malaria parasite (Rayner *et al.* 2011).

P. vivax is discussed to be the most prevalent malaria parasite globally. Infections with *P. vivax* were described in bonobos, all four chimpanzee subspecies, and eastern and western gorillas (summarised in Sharp *et al.* (2020)). The closest relative seems to be *Plasmodium carteri* parasitising chimpanzees in Africa.

P. malariae closely resembles *Plasmodium rodhaini* in African apes and *Plasmodium brasilianum* in simian New World monkeys not only morphologically but also genetically (summarised in Sharp *et al.* (2020)). Both primate parasites can infect humans (Lalremruata *et al.* 2015). Moreover *malariae*-like parasites were documented in chimpanzees.

Both, *P. ovale curtisi* and *P. ovale wallikeri* have close relatives in free-ranging apes (summarised in in Sharp *et al.* (2020)). *Curtisi*-like parasites were documented in chimpanzees and bonobos, and *wallikeri*-like in gorillas. Moreover, *P. ovale wallikeri* was documented in the faeces of Western lowland gorillas in the Central African Republic (Mapua *et al.* 2018).

P. knowlesi is a simian malaria parasite which distribution is limited to South-Eastern Asia. The distribution area is limited by those of its vector – mosquitoes of the *Anopheles leucocsphyrus*-group. Intermediate hosts are long tailed macaques (*Macaca fascicularis*), pig tailed macaques (*Macaca nemestrina*), and mitered leaf monkeys (*Presbytis melalophos*). In its natural hosts infections are reported to be mild, but documented to be severe if experimentally inoculated to other primates, e.g. rhesus monkeys (Coatney *et al.* 1971, Cox-Singh and Singh 2008). This parasite gained relevance when it was discovered as causative of a large malaria outbreak in humans in Malaysian Borneo using molecular diagnostic tools (Singh *et al.* 2004).

Various vector-borne filaroid parasites are known to infect non-human primates. Of those only some are known to be transmitted by mosquitoes.

The human parasites *Brugia malayi* and *Brugia pahangi* were described in various mammals including *Macaca* spp. (summarised in Votypka *et al.* (2020). Leaf-eating monkeys (*Presbytis* spp.) are discussed to be main reservoir hosts for *B. malayi* (Irwin and Jefferies 2004). Adult helminths are located in the lymphatic and peri-lymphatic tissues. Dogs and cats are reservoir hosts for *B. malayi*, but are the main hosts for *B. pahangi*. *B. malayi* is endemic in South-East Asia, China, and a small area in southwestern India (Tolle 2009). Although microfilariae were documented in various mosquito genera, *Mansonia* are documented as main vectors (WHO 2013). Mosquitoes of the genera *Mansonia* are known as natural vectors of *B. pahangi*, but *Armigeres subalbatus* seem to be the most relevant vector in urbanised areas (Muslim *et al.* 2013).

Various species of the genus *Dirofilaria* are reported to infect non-human primates. *Dirofilaria corynodes* is the most prevalent filarioid helminth in Old World monkeys in Africa (e.g. guenons, mangabeys, and patas monkeys). *D. corynodes* are large parasites (females up to 29 cm) found in sub-cutaneous tissue of lower extremities and the trunk. In patas monkeys imported to the USA from Nigeria 12 of 211 patas monkeys (*Erythrocebus patas*) were positive for this parasite, presenting unsheated microfilariae in the peripheral blood with nocturnal periodicity (Orihel

1969). Under laboratory conditions larval development was demonstrated in *Aedes aegypti* mosquitoes (Orihel 1969, Travi *et al.* 1986).

Dirofilaria magnilarvatum was reported from long-tailed macaques (*M. fascicularis*) in Asia. Parasites were found in the subcutaneous connective tissue with females reaching a size of up to 9 cm (Price 1959). Microfilariae are extremely large (580 µm long). Mosquitoes of the genus *Mansonia* were described as competent vectors (Wharton 1959).

Mosquito-borne diseases in carnivores

Viruses

Although their role in the transmission of mosquito-borne diseases has not been explored extensively; carnivores can also be hosts of arboviruses. Mesocarnivores, such racoons (*Procyon lotor*) and skunks (*Mephitis mephitis*), have shown high prevalence of antibodies against the West Nile Virus in North America (Root and Bosco-Lauth 2019), while antibodies have been detected in red foxes in Spain (Gutiérrez-Guzman *et al.* 2012). However, it remains to be understood if the viremia present in these mammals is enough to consider them as amplifying hosts. Captive and wild bears (*Ursus* sp.) show severe clinical manifestation for WNV infection (Root and Bosco-Lauth 2019). The Murray Valley encephalitis virus (Togaviridae) causes clinical manifestations like those by the Japanese encephalitis virus. This virus is endemic from Australia and New Guinea and has been isolated from several mammals species including foxes (Odend'hal 1983).

Parasites

The most relevant mosquito-borne parasites in carnivores are filarioid helminths. As explained above, most information in wildlife is present for those parasites which are of relevance for human and/or veterinary health.

Up to the current knowledge, all species of the genus *Dirofilaria* (family Onchocercidae) are transmitted by mosquitoes. The only exception is *Dirofilaria ursi* which is transmitted by black flies (Simulidae) (Dantas-Torres and Otranto 2013). *Dirofilaria immitis*, also called canine heartworm, is the causative agent of canine (and feline) cardiopulmonary dirofilariosis. *D. immitis* is distributed in tropical, subtropical, and temperate regions worldwide. It is transmitted by mosquitoes of various genera including *Aedes, Culex* and *Anopheles* (Simon *et al.* 2012). Dogs are the definitive hosts, but cats can also harbour this parasite (Simon *et al.* 2012). Infections in various wild carnivores but also humans were reported. In less suitable hosts parasites do not develop to adults and are so not able to shed microfilaria.

In Northern America microfilaremic infections were documented in coyotes (*Canis latrans*) and red foxes (*V. vulpes*). Coyotes show high prevalences with *D. immitis* infections. Infections were also reported in red wolves and their hybrids (reviewed in (Simon *et al.* 2012)). In Southern America *D. immitis* was reported in the maned wolf (*Chrysocyon brachyurus*) in Bolivia. In Australia *D. immitis* was found in red foxes in areas surrounding Melbourne (Marks and Bloomfield 1998). Furthermore *D. immitis* was reported from Felidae, like ocelots, jaguars, lions, tigers, cougars, a snow leopard, and leopards, but also in Ursidae like black bears (summarised in Simon *et al.* (2012) and Murata *et al.* (2003)). Moreover it was documented in raccoons (*Procyon lotor*) (Snyder *et al.* 1989). In Europe *D. immitis* was also documented in wild cats (*Felis silvestris*), but also in red foxes and golden jackals

(*Canis aureus*), grey wolves (*Canis lupus*), brown bear (*Ursus arctos*), and Eurasian otters (*Luttra luttra*) (Ionica *et al.* 2017, Papadopoulos *et al.* 2017)).

Dirofilaria repens is the causative agent of subcutaneous dirofilariosis in dogs. Like *D. immitis* it can also infect various carnivores, but also humans (Simon *et al.* 2012). Mosquitoes of the genera *Aedes, Culex* and *Anopheles* are intermediate hosts and vectors of this pathogen (Capelli *et al.* 2018). In Europe *D. repens* was reported in red foxes (*V. vulpes*), golden jackals (*C. aureus*), grey wolves (*C. lupus*), beech marten (*Martes foina*), and a least weasel (*Mustela nivalis*) (summarised in Ionica *et al.* (2017)).

Dirofilaria striata was described as parasite of bobcats (*Lynx rufus*) and Florida panthers in the USA. It was also reported from the puma (*Felis concolor*) in Brazil and Florida, and the ocelot (*Felis pardalis*) in Venezuela (summarised by Orihel and Isbey (1990)). Genetically they are more similar to *D. immitis* than *D. repens* (Wyatt *et al.* 2020). Moreover, it was documented in domestic cats and a human where it was removed from the orbit (Orihel and Isbey 1990, Wyatt *et al.* 2020). Anopheline mosquitoes were discussed as vectors. The parasite can be distinguished from other *Dirofilaria* species by its large size and unique cuticular structure (Orihel and Isbey 1990).

Dirofilaria lutrae is a subcutaneous parasite of otters like the North American river otters (*Lontra canadensis*) in the USA (Orihel 1965, Swanepoel *et al.* 2018). It was also documented in mink (*Neovison vison*) from Florida. Phylogenetically it is closely related to *D. immitis*.

Dirofilaria spectans parasitise in the cardiac cavities of the giant otter (*Pteronura brasiliensis*), neotropical river otter (*Lontra longicaudis*) in Brasil (Soto 2000). Female nematodes have a size up to 20 cm.

Dirofilaria tenuis is a filarioid parasite presenting subcutaneous nodules in raccoons in the Southeastern USA and is reported as the most common zoonotic filaria in the USA (Vincent *et al.* 2013). The black salt marsh mosquito (*Aedes taeniorhynchus*) has been incriminated as a vector.

Dirofilaria cancrivori was documented in the subcutaneous tissues of the crabdog (*Procyon cancrivorus*) in Guyana, South America (Eberhard 1978). Various other *Dirofilaria* species have been reported in carnivores of which most were reported in pre-molecular diagnostic times. Dantas-Torres and Otranto (2013) summarised those of valid species – *Dirofilaria ailure* in Procyonidae in China, *Dirofilaria acutiuscula* in Canidae and Felidae, *Dirofilaria genettae* in Felidae and Viverridae in Nigeria, *Dirofilaria granulosa* in Felidae in Africa and Asia, *Dirofilaria minor* in Felidae in Vietnam, *Dirofilaria pagumae* in Viverridae in Indochina, and *Dirofilaria sudanensis* in Felidae and Hyaenidae in Sudan. However, molecular techniques have allowed the description of *Candidatus Dirofilaria hongkongensis*, and documented high genetic diversity of *D. repens* (*D. repens* species complex) in Asia (Yilmaz *et al.* 2019).

B. pahangi is closely related to *B. malayi* and was documented in cats, dogs, wild carnivores, and was recently reported in humans in Malaysia (Tan *et al.* 2011). A co-infection of *D. immitis* with *B. pahangi* was documented in a Malaysian clouded leopard, *Neofelis nebulosa* (Zahedi *et al.* 1986).

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3. Diptera-borne hemoparasites of herpetofauna: rediscovering its importance

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Abstract

A great variety of microorganisms need dipterans as part of their life cycle. The dominance of these insects as vectors is differential between vertebrates; thus, they have a leading role in transmitting viruses, protozoans, and other pathogens to mammals and birds; however, they have a more discrete representation as vectors of parasites among ectotherms. Most of the parasites that affect herpetofauna are transmitted mainly by mites, ticks, leeches. At the same time, in less proportion use generalist or herpetophilic dipterans, which have developed strategies to extend their feeding sources to warm-blooded vertebrates. On the other hand, hemoparasites belonging to Apicomplexa, Trypanosomatida, and Spirurida have generated mechanisms for dissemination other than the infection by bite. This chapter aims to provide a detailed review of the current knowledge of the hemoparasites of herpetofauna transmitted by dipterans, collecting classic literature until recent research. We also discuss the life cycle transmission of these parasites and their possible implications in public health scenarios.

Keywords: vector, reptile, amphibian, transmission, sandflies, mosquitoes, blood parasites, cold-blooded vertebrates

Introduction

Hemoparasites

Parasites represent about 50% of the existing species in the world. They are related to the emergence and re-emergence of infectious diseases and are recognised as a zoonotic pool, affecting domestic animals, wildlife, and humans (Daszak *et al.* 2000). Overall, parasites exhibit regulatory functions in the ecosystem based on a series of complex interactions that involve coexistence, competition, or predation, which do not necessarily cause adverse or pathological effects in their hosts (Bower *et al.* 2019, Hatcher *et al.* 2012).

Reptiles and amphibians are affected by different viral, bacterial, and fungal diseases. Furthermore, like other taxonomic groups, they are not exempt from infections caused by Nematoda and Protozoa; in fact, the herpetofauna is commonly infected with Apicomplexa blood protozoa as well as Euglenozoa parasites.

Compared with the knowledge of the life cycles of parasites that infect endotherms, particularly mammals, the information available about blood parasites of reptiles and amphibians is scarce and fragmented. Only a few authors have described their complete life cycles and published

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detailed information. The most comprehensive publications about these subjects are (1) 'Malarial parasites and other Haemosporida' (Garnham, 1966), covering saurian, avian, and mammalian malaria parasites; (2) 'A contribution to the systematics of the reptilian malaria parasites, family Plasmodiidae (Apicomplexa Haemospororina)' (Telford, 1988) and (3) 'Haemoparasites of reptiles' (Telford, 2009), compile morphological descriptions of parasites, their vertebrate hosts, and potential vectors found worldwide; (4) Schall (1990, 1996, 1986) added basic information on parasites' biology in reptiles; (5) Perkins (2001) and Falk *et al.* (2011) published valuable knowledge about blood parasites of lizards in the Antilles; and (6) Lainson (2012) published an atlas with detailed draws of parasite stages and their hosts found in the Brazilian herpetofauna, mainly in the Amazonian basin.

The phylum Apicomplexa are obligate intracellular parasites, which include around 6,000 species to date. Within this group, there are parasites with a significant impact on public health, such as *Toxoplasma, Cryptosporidium*, and *Plasmodium* (Adl *et al.* 2019, 2007). Two taxonomic groups represent the majority of blood parasites found in herpetofauna; they are Coccidia and Haemosporida.

Among Coccidia, the parasites found infecting reptiles and amphibians, commonly known as hemogregarines, belong to the suborder Adeleorina. Within this taxonomic category are included *Haemogregarina*, *Babesiosoma*, *Hemolivia*, restricted to turtles and tortoises; *Hepatozoon,* very common in snakes and amphibians, *Dactylosoma* restricted to amphibians, and *Karyolysus* found in lizards and amphibians (Cook *et al.* 2016, Haklová-Kočíková *et al.* 2014, Lehmann 1959). In addition, the coccidian genera *Schellackia, Lainsonia,* and *Lankesterell*a of the subgenus *Eimeriorina* have also been reported infecting herpetofauna. However, they are less frequent and have been less studied than the Adeleorina parasites. The level of conservation of a particular ecosystem may influence infection characteristics by blood parasites in lizard species, e.g. *Psammodromus algirus* (a Mediterranean lizard) showed high prevalence and parasitemia caused by *Schellackia* sp. in a deteriorated habitat (lowland holm oak forest), while in a better-preserved environment, the prevalence and parasite load were lower (well-preserved mountain oak forest) (Carbayo *et al.* 2018); similar results were reported by (Amo *et al.* 2007) in the same lizard species.

A wide range of arthropods transmits blood parasites in ectotherms (Siddall and Desser 1991, Smith 1996, Telford 2009). However, some of them act as passive vectors. When the vertebrate host ingests them, it becomes infected with the parasite carried by the vector (e.g. *Schellackia* and *Culex pipiens fatigans* (Lainson *et al.* 1976)). Transmission by a bite of an infected invertebrate is just one of at least three mechanisms by which amphibians and reptiles can acquire haemoparasite infections (Kauffman *et al.* 2017, Landau *et al.* 1972, Murata *et al.* 1993, Siddall and Desser 1991, Telford 2009). This review mainly focuses on parasites that use blood-sucking dipterans to accomplish a part of their life cycle or as vehicles for their transmission to the reptile or amphibian hosts. In this way, we will deal in detail with Coccidians like *Dactylosoma, Hepatozoon, Schellackia, Lainsonia,* and *Lankesterella*; the Haemosporidian genera *Plasmodium, Garnia,* and *Haemocystidium,* and their potential or confirmed vectors (Barta 1991, Cook *et al.* 2015, Lainson 2012, Telford 2009, Tomé *et al.* 2014). Also, common blood parasites belong to the phylum Euglenozoa, order Kinetoplastida of the genera *Trypanosoma* and *Leishmania* (Brygoo 1963a, Killick-Kendrick *et al.* 1986, Ovezmukhammedov 1991, Rasoanoro *et al.* 2020), and Nematoda of the superfamily Filaroidea reported in amphibians and reptiles that may be transmitted by Diptera insects (García-Prieto *et al.* 2014).

Co-occurrence of the parasite, the insect vector, and the vertebrate host is strictly necessary to perpetuate the parasite (Ferguson and Smith 2012). Although the blood parasites discussed in this chapter have life cycles that involve Diptera as vectors, it is essential to highlight that given the diversity of herpetofauna's habitats (many of them spend part of their biological cycle in aquatic environments), other vectors such as leeches, ticks, and mites are commonly implied. Likewise, it is common for vertebrate hosts to ingest the infected insect and thus develop an infection; an example is *Hepatozoon* parasites in amphibians (Smith 1996). Other transmission mechanisms, such as predation of paratenic hosts or even infection by free sporozoites in water, have also been identified (Davies and Johnston 2000).

Mechanism of transmission to hosts

There are some forms in which arthropods can transmit parasites to vertebrate hosts, and for that reason important to know about the biology of parasites, the biology, habits, and ecology of hosts, and the biology, feeding mechanisms, habits, and ecology of the arthropods that may function as vectors. This information makes it possible to advance in the epidemiology of the diseases caused by a specific parasite (Figure 1).

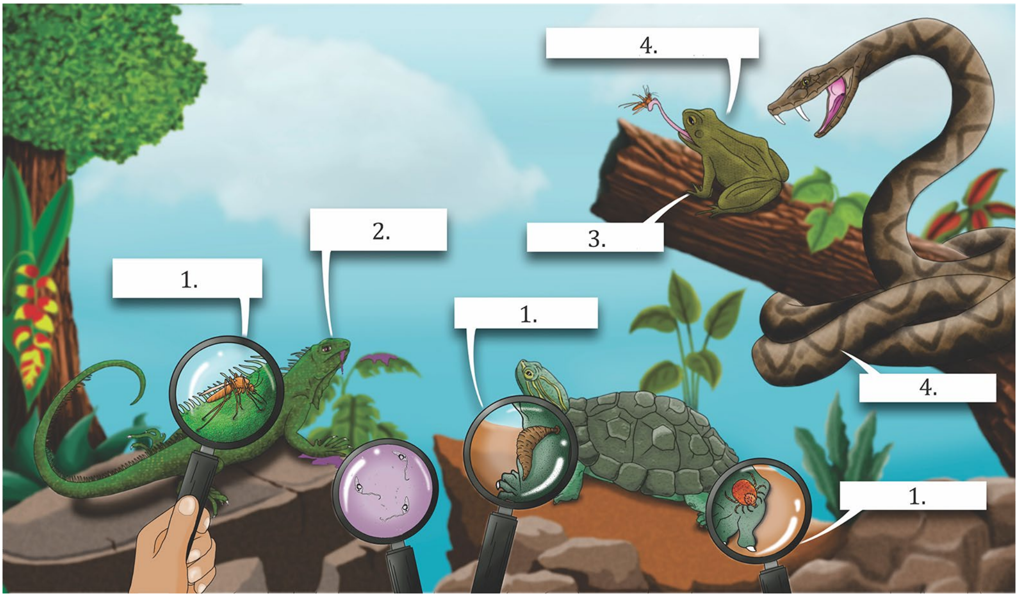

Figure 1. Transmission mechanisms used by blood parasites in herpetofauna. Biological transmission by bites of infected insects (1) has been reported in the life cycle of Plasmodium *sp.,* Haemocystidium *sp.,* Leishmania *sp.,* Trypanosoma *sp., and* filaria, *which are transmitted by mosquitoes; for* Haemogregarina *sp.,* Trypanosoma *sp.,* Dactylosoma *sp., vectored by leeches; and* Hemolivia *sp.,* Trypanosoma *sp., and* filaria *transmitted by ticks.* Trypanosoma *sp. can also be transmitted when the contaminated faeces of the vector comes in contact with the wound produced by the insect at the time of feeding (2). The ingestion of a vector or an infected host leads to the mechanical transmission of* Schellackia *sp. and* Lainsonia *sp. (3), while biological transmission of parasites like* Hepatozoon, *some species of* Trypanosoma, *and* Leishmania (Sauroleishmania*) can be accomplished through this mechanism (4).*

Vertebrates may acquire parasites and pathogens with the participation of arthropod vectors by the following general mechanisms (James and Harwood 1969, Lane and Crosskey 1993):

1. Mechanical transmission. The parasite does not need to pass any stage of development or multiplication in the arthropod.
    a. The infected arthropod spreads the parasite by contact with contaminated body parts or excretion with potential hosts (active).
    b. Entomophagous vertebrate feeding on infected arthropods (Wenyon 1926) (passive).
2. Biological transmission. The parasite or pathogen requires the vector to complete its life cycle, like a stage of development or multiplication in the arthropod. Different mechanisms may produce this type of transmission:
    a. The parasite (which needs to pass part of its life cycle in the arthropod) is transmitted when the vertebrate eats the infected arthropod.
    b. The parasite or pathogen is transmitted directly by infected arthropods that feed on the blood of susceptible hosts (Wallace 1966) (active, from the vector point of view).

Despite all these possibilities of transmission, the most important mechanism of parasite transmission by arthropods is hematophagy, and some frogs' behaviour seems to avoid being infected by mosquitoes, such as stomping, jumping and cleaning their bodies with their feet to scatter mosquitoes (Crans 1970, Ferguson and Smith 2012). The relationship between mosquitoes and amphibians does not occur in a unidirectional way like other predator-prey relationships. Instead, there is a reciprocal nature in the relationship between hematophagous Diptera and amphibians, in which several species of hemoparasites employ mechanical or salivary transmission (Ferguson and Smith 2012).

Hematophagous Diptera associated with the transmission of hemoparasites of herpetofauna

Hematophagy refers to the nutritional habit of ingesting vertebrate blood to obtain proteins and lipids. There are some groups of animals that acquired this feeding habit independently over millions of years. However, one of the most important is the arthropods, including mites, ticks, and insects (García *et al.* 2015).

Independently of the annoyance of their bites, allergy, or other types of reactions, hematophagous arthropods are important because they may transmit pathogens to their hosts. Some viruses, bacteria, Protozoa, Nematoda, and Platyhelminthes are transmitted to vertebrate hosts.

Some arthropods evolved to take advantage of blood as food. Some live at the expense of this as a unique resource. Others need blood just for oogenesis and to improve the fitness of their brood. The main arthropod groups with hematophagous species are Class Arachnida, the Order Acari and the Class Insecta, the Order Phthiraptera, Order Hemiptera (Cimicidae Reduviidae: Triatominae, and Polyctenidae), Order Siphonaptera, and the members of some families of Order Diptera (Grimaldi *et al.* 2005). Some groups have received particular attention, including vectors of pathogens that affect human and domestic animal health. However, Diptera stands out as they are responsible for transmitting pathogens that produce epidemic diseases with high mortality in humans and domestic animals (Lane and Crosskey 1993).

Thirteen Diptera families include hematophagous species: Psychodidae, Simuliidae, Ceratopogonidae, Corethrellidae, Culicidae, Athericidae, Tabanidae, in which only females take blood for reproduction, and Muscidae, Calliphoridae, Nycteribiidae, Streblidae, and Hippoboscidae, of which the two sexes feed on blood (Ibáñez-Bernal *et al.* 2020). Records of hematophagy on amphibians and reptiles occur within families Psychodidae, Ceratopogonidae, Corethrellidae, Culicidae, Athericidae, Tabanidae, and Glossinidae; the other families mentioned include species that feed on hot-blooded vertebrates and will not be treated here.

Psychodidae (sandflies). Among the six extant subfamilies (Wagner and Ibáñez-Bernal 2009), two subfamilies include species with hematophagous habits, Phlebotominae and Sycoracinae may be vectors of pathogenic agents (Young 1979). Females of both subfamilies have piercing mouthparts with fully developed mandibles (Duckhouse 1972, Young 1979), allowing them to suck vertebrate blood. Poinar Jr and Poinar (2004) found in *Palaeomyia burmitis* Poinar, (a species from Early Cretaceous Burmese amber 105-100 mya), amastigotes, and promastigotes' Leishmania like demonstrating that the relation between Sauroleishmania-sand flies and reptiles is old.

Subfamily Sycoracinae is represented by *Sycorax* Haliday in Curtis. However, Duckhouse (1972) split it into three genera: *Sycorax*, *Aposycorax* Duckhouse, and *Parasycorax* Duckhouse, with about 34 extant species worldwide (Bravo and Salazar-Valenzuela 2009, Dos Santos *et al.* 2009). Feeding preferences of most species of Sycoracinae are still unknown, except by *Sycorax silacea* Curtis and *Sycorax wampukrum* Bravo and Salazar-Valenzuela, which were collected feeding on frog blood. *S. silacea* Haliday, was also related to the transmission of a filarial worm (Chikhlyaev *et al.* 2019, Desportes 1942), demonstrating the potential importance of these species in the transmission of parasites to amphibians.

Subfamily Phlebotominae includes about 1000 described species worldwide (Shimabukuro *et al.* 2017). Females of nearly all species require ingestion of vertebrate blood to make possible the oogenesis and improve the brood's fitness. There are few records of species that feed on amphibians, reptiles, and opportunistic on other vertebrates. However, most information concerns those species associated with various mammals and birds (Young and Duran 1994), which can occasionally feed on amphibian and reptiles' blood (Lewis 1978). The phylogenetic classification of Galati, (1995) recognises about 30 genera worldwide, of which at least *Sergentomyia (Se)* França and Parrot, *Micropygomyia* Barretto and *Lutzomyia* (*Helcocyrthomyia*) Barretto, include species that feed on cold-blooded vertebrates, few of which also have been found naturally infected with trypanosomatids and filarial worms (Lane 1993, Tesh *et al.* 1971).

Ceratopogonidae (biting midges). About 6,267 described valid extant and extinct species in 133 genera worldwide. (Borkent *et al.* 2018). Nevertheless, it is a group with different feeding habits, from adults that do not feed to those that feed on nectar and species with females that suck hemolymph of other arthropods. Genera *Culicoides* Latreille, with more than 1,000 species (Borkent 2016a), *Leptoconops* Skuse, with about 133 extant species (Borkent 2016b), *Austroconops* Wirth and Lee, with one extant species, and *Forcipomyia* (*Lasiohelea*) Kieffer with 182 species worldwide, include species with females that suck vertebrate blood (Borkent 2009, 2004, Borkent and Dominiak 2020, Ibáñez-Bernal *et al.* 2020). There is evidence that *Forcipomyia* (*Lasiohelea*) can transmit *Icosiella neglecta* to frogs (Boorman 1993, Chikhlyaev *et al.* 2019). Many species of *Culicoides* are proven vectors of viruses, several protozoans, and filarial worms to vertebrates. However, a study on the blood-feeding preferences on ectotherms of some species must be investigated yet.

Corethrellidae (frog-biting midges). This family includes over 97 extant species belonging to the genus *Corethrella* Coquillett, distributed principally in tropical and subtropical regions (Borkent 2008). Females have biting mouthparts and are attracted to frogs' mating calls (Bernal *et al.* 2006, Borkent 2009, McKeever 1977). There is a record related to the transmission of trypanosomes among frogs (Johnson *et al.* 1993). Studies about the family are scarce, and more studies are needed to demonstrate their importance as vectors of frog parasites.

Culicidae (mosquitoes) are probably one of the best-studied families of Diptera because it includes species responsible for the transmission of diseases of human, domestic, and wild vertebrates. 3,583 extant species have been described and classified in two subfamilies and 113 genera (Harbach 2008). Females of most species are hematophagous, some do not have preferences for hosts, and others show some specificity in its selection; some feed on mammals, others on birds, but some species are known to feed on amphibians and reptiles (Chaverri 2009, Tempelis 1975). We can mention the works of Reeves *et al.* (2019) in which *Culex atratus* Theobald and *Culex pilosus* (Dyar and Knab) were narrowly associated with reptilian host species indicating they are specialists of reptilian hosts, particularly *Anolis* lizards, Tempelis (1975) which indicated that *Culex territans* Walker, feed almost exclusively on amphibians, *Deinocerites dyarii* Belkin and Hogue, that feeds predominantly on reptiles but will take an occasional blood meal from a homeothermic animal, and *Deinocerites epitedeus* (Knab) that readily feed on both poikilothermic and homeothermic animals. Snyman (2020) made an extensive revision of mosquitoes that feed on amphibians and reptiles, indicating their habit for *Uranotaenia* Lynch Arribálzaga spp., *Mimomyia* Theobals spp. Some species of *Culex* (*Melanoconion*) Theobald, *Culex* (*Afroculex*) Danilov, some of *Culex* (*Culex*) Linnaeus, and other few species of *Aedeomyia* Theobald, *Aedes* Meigen, *Anopheles* Meigen, *Coquillettidia* Dyar, *Mansonia* Blanchard, that sometimes also feed on amphibians or reptiles. Some have been proposed as vectors of ectotherm parasites (Table 1).

Athericidae (Athericid flies). About 100 species in ten genera have been described for the family around the world, of which about 47 species belong to the genus *Suragina* Walker (Rafael and Henriques 1990, Stuckenberg 2000), some known as blood feeders on frogs, birds, and mammals (Woodley 2009), but with no evidence of pathogen transmission to hosts. The biology of these species is poorly known.

Tabanidae (deer flies, horse flies). This family includes about 4,406 named species worldwide (Borkent *et al.* 2018). Females of a few species may be autogenous, but most of them feed on the blood of mammals and few species on the blood of reptiles and birds (Fairchild 1986, Middlekauff and Lane 1980). Information about species feeding on reptiles is rare. However, the following species are known with this habit: *Esenbeckia* Rondani (Cupul-Magaña *et al.* 2017) feeding on crocodiles, *Fidena* Walker (Fairchild 1986), *Catachlorops* Lutz, *Diachlorus* Osten Sacken, *Lepiselaga* Macquart, *Phaeotabanus* Lutz, *Stenotabanus* Lutz, and *Tabanus* Linnaeus, parasitising caimans (Tellez 2013). Some species of tabanids have been linked to the mechanical transmission of parasites in crocodiles and caimans (Barros 1996, Ferreira *et al.* 2002, Hoare 1929, Onmaz *et al.* 2013).

Glossinidae (Tsetse flies). There are more than 23 distributed across sub-Saharan Africa, and all belong to the genus *Glossina* Wiedemann. The adults are blood feeders on reptiles, birds, and mammals. Some species are the biological vectors of several *Trypanosoma* species. Several species and subspecies feed on reptiles, for example, *Glossina fuscipes fuscipes* (Newstead), *Glossina palpalis gambiensis* Vanderplank, *Glossina palpalis palpalis* (Robineau-Desvoidy), *Glossina longipalpis* Wiedemann, *Glossina medicorum* Austen and, *Glossina tachinoides* Westwood (Hoppenheit *et al.* 2013, Snyman 2020, Späth 2000).

Table 1. Parasite species, their hosts, and Diptera vectors. Countries and references of the studies where dipterans were identified as vectors (or potential) are provided.

Species	Vertebrate host	Vector	Country	Reference
Apicomplexa: Lankestellidae				
Schellackia sp.				
Schellackia landaue	Polychrotidae: *Polychrus marmoratus*	*Culex pipiens fatigans*	Brazil	Lainson et al. 1976
Apicomplexa: Haemogregarinidae				
Hepatozoon sp.				
Hepatozoon catesbianae	Ranidae: *Lithobates catesbeianus*	*Culex territans*[1]		Desser *et al.* 1995
Hepatozoon clamatae	Ranidae: *Lithobates clamitans*	*Cx. territans, Cx. pipiens*[1]		Harkness *et al.* 2010
Hepatozoon rarefaciens	Colubridae: *Drymarchon corais*	*Culex tarsalis*[1], *Anopheles albimanus*[1], *Aedes sierrensisa*[1]		Ball and Oda, 1971
Hepatozoon ayorgbor	Pythonidae: *Python regius*	*Culex quinquefasciatus*[1]	Ghana	Sloboda *et al.* 2007
Hepatozoon caimani		*Cx. quinquefasciatus*[1]		Paperna and Lainson 2003
Hepatozoon tupinambis	Tupinambis teguixin	*Culex fatigan*[1]	Brazil	Pessôa *et al.* 1974
Hepatozoon domerguei	Colubridae: *Madagascarophis colubrinus*	*Cx. quinquefasciatus, Anopheles stephensi*[1]	Madagascar	Landau *et al.* 1972
Hepatozoon fusifex	Boidae*: Boa constrictor*	Cx. tarsalis, Aedes dissimile	Mexico	Ball *et al.* 1969
Hepatozoon affluomaloti	Cordylidae: *Pseudocordylus melanotus*	*Culex (Afroculex) lineata*[1]	South Africa	Van As *et al*. 2015
Hepatozoon aegypti	Colubridae: *Spalerosophis diadema*	*Cx. pipiens molestus*[1]	Egypt	Bashtar *et al.* 1984
Hepatozoon terzii	Boidae: *B. constrictor*; Tropiduridae: *Tropidurus torquatus*	*Cx. quinquefasciatus*[2]		Paperna and Lainson 2004
Hepatozoon horridus	Viperidae: *Crotalus horridus*	*Aedes aegypty*[1]	USA	Telford *et al.* 2008
Hepatozoon sauritus	Viperidae: *C. horridus*	*Ae. aegypty*[1]	USA	Telford *et al.* 2008
Hepatozoon sipedon	Colubridae: *Nerodia sipedon*	*Cx. pipiens, Cx. territans*	Canada	Smith *et al.* 1994
Dactylosoma sp.				
Dactylosoma ranarum	Ranidae: *Pelophylax esculentus*	*Culicoides nubeculosus*		Netherlands *et al.* 2020a
Dactylosoma kermiti	Bufonidae: *Sclerophrys gutturalis*; Ptychadenidae: *Ptychadena anchietae*	*Uranotaenia mashonaensis*		Netherlands *et al.* 2020a
Apicomplexa: Haemoproteidae				
Haemocystidium sp.				
Haemocystidum degiustii (syn. *Haemoproteus 'metchnikovi*)	Chelidae: *Chrysemys picta marginata*	*Chrysops callidus*[2]	USA	De Giusti *et al.* 1973

Table 1. continued.

Species	Vertebrate host	Vector	Country	Reference
Apicomplexa: Plasmodidae				
Plasmodium sp.				
Plasmodium agamae	Agamidae: *Agama agama*	*C. nubeculosus*	Sudan	Petit *et al.* 1983
Plasmodium floridense	Anolis: *Dactyloidae*; Sceloporus: *Phrynosomatidae*	*Ae. aegypti*[2], *Culex erraticus*[2], *Cx. territans*[2]	USA	Huff 1941, Klein *et al.* 1987
Plasmodium giganteum	Agamidae: *A. agama*	*Ae. aegypti, Aedes simpsoni, Aedes apicoargenteus, Aedes africanus*	Liberia	Baker 1961
Plasmodium mexicanum	Phrynosomatidae: *Sceloporus torquatus*	*Lutzomyia vexator*[2], *Lutzomyia stewarti*[2]	Mexico	Ayala and Lee 1970, Ayala 1971a, Klein 1985
Saurocytozoon sp.				
Saurocytozoon tupinambi	Teiidae: *T. teguixin*	*Cx. pipiens*	Brazil	Lainson and Shaw 1969
Apicomplexa: Garniidae				
Fallisia sp.				
Fallisia effusa	Gymnophthalmidae: *Neusticurus bicarinatus*	*Phlebotomine* sandflies	Brazil	
Garnia sp.				
Garnia morulum (*Plasmodium morulum*)	Scincidae: *Mabuya mabouya*	*Cx. pipiens*	Panama	Lainson *et al.* 1974
Euglenozoa: Trypanosomatidae				
Trypanosoma sp.				
Trypanosoma sp.	Amphibia or Reptilia	*Phlebotomus kazeruni*[2]	Pakistan	Kato *et al.* 2010
Trypanosoma thecadactylii	Gekkonidae: *Thecadactylus rapicaudus*	*Lutzomyia trinidadensis*[1]	Panama	Christensen and Telford 1972
Trypanosoma scelopori	Phrynosomatidae: *Sceloporus occidentalis*	*Lutzomyia vexator occidentis*[1]	USA	Ayala 1970
Trypanosoma gerrhonoti	Anguidae: *Gerrhonotus m. multicarinatus*	*L. vexator occidentis*[1]	USA	Ayala and McKay 1971
Trypanosoma bufophlebotomi	Bufonidae: *Bufo boreas halophilus*	*L. vexator occidentis*[1]	USA	Ayala 1971b
Trypanosoma boueti	Scincidae: *Mabuya raddoni*	*Sergentomyia bedfordi*[1]	French Guinea	Ashford *et al.* 1973
Trypanosoma grayi	Crocodylidae: *Crocodylus niloticus*	*Glossina palpalis*[2], *Glossina tachinoides*[2]	Uganda	Novy 1906, Hoare 1928, 1929, 1931a,b
Trypanosoma platydactyli	Gekkonidae: *Tarentola mauritanica*	*Sergentomyia minuta*[2]	Tunisia	Adler and Theodor 1935
Trypanosoma phlebotomi	Gekkonidae: *Hemidactylus frenatus*	*Sergentomyia babu shortti*[2]	India	Shortt and Swaminath, 1931

Table 1. continued.

Species	Vertebrate host	Vector	Country	Reference
Euglenozoa: Trypanosomatidae				
Trypanosoma rotatorium	Ranidae: *Rana clamitans*	*Cx. territans*[2], *Ae. aegypti*[2]	Canada	Desser *et al.* 1973
Trypanosoma kaiowa	Alligatoridae: *Caiman yacare*	*Phaeotabanus fervens*[2]	Brazil	Fermino *et al.* 2019
Trypanosoma cascavelli	Viperidae: *Crotalus durissus*	*Viannamyia tuberculata*[2]	Brazil	Viola *et al.* 2008
Leishmania (*Sauroleishmania*) sp.				See Tichá 2019
Nematoda: Onchocercidae				
Conispiculum sp.				
Conispiculum flavescens (syn. *Cx. guindiensis*)	Agamidae: *Calotes versicolor*	*Cx. quinquefasciatus*[1]	India	Pandit *et al.* 1929
Oswaldofilaria sp.				
Oswaldofilaria bacillaris	Alligatoridae: *Caiman crocodilus*	*An. stephensi*	Brazil	Prod'hon and Bain 1972
Oswaldofilaria belemensis	Teiidae: *Dracaena guyanensis*	*Ae. aegypti*[1]	South America	Bain and Chabaud 1975
Oswaldofilaria chlamydosauri	Agamidae: *Amphibolurus barbatus*	*Culex annulirostris*[1], *Cx. quinquefasciatus*[1]	Austrailia	Mackerras 1953
Oswaldofilaria petersi	Teiidae: *Tupinambis nigropunctatus*	*Cx. quinquefasciatus*[1], *Cx. pipiens*[1]	Brazil	Bain and Chabaud 1975
Oswaldofilaria spinosa	Scincidae: *Mabuya mabouia*	*Cx. quinquefasciatus*[1], *Cx. pipiens*[1]	Brazil	Bain and Chabaud 1975
Icosiella sp.				
Icosiella neglecta	Ranidae: *Rana esculanta*	*Forcipomyia velox*[2], *Sycorax silacea*[2]	Europa and North Africa	Desportes 1941, 1942
Foleyellides sp.				
Foleyellides brachyoptera (syn. *Waltonella brachyoptera*)	Ranidae: *Rana sphenocephala*	*Cx. quinquefasciatus*[1], *Cx. pipiens*[1]	USA	Kotcher 1941
Foleyellides dolichoptera (syn. *Waltonella dolichoptera*)	Ranidae: *R. sphenocephala*	*Cx. pipiens*[1], *Cx. quinquefasciatus*[1]	USA	Kotcher 1941
Foleyellides flexicauda (syn. *Waltonella flexicauda*)	Ranidae: *Rana catesbeiana*	*Cx. territans*[2]	USA	Benach and Crans 1973
Foleyellides ranae (syn. *Waltonella ranae*)	Ranidae: *R. catesbeiana*	*Ae. aegypti*[1], *Cx. quinquefasciatus*[1], *Cx. pipiens*[1]	USA	Causey 1939a,b,c, Kotcher 1941
Foleyellides duboisi	Ranidae: *Rana esculenta ridibunda*	*Culex molestus*[1]	North Palestine	Witenberg and Gerichter 1944

Table 1. continued.

Species	Vertebrate host	Vector	Country	Reference
Nematoda: Onchocercidae				
Foleyellides sp.				
Neofoleyellides sp.				
Neofoleyellides boerewors	Bufonidae: *Sclerophrys gutturalis*	*U. mashonaensis*[2], *Uranotaenia montana*[2]	South Africa	Netherlands *et al.* 2020b
Foleyella sp.				
Foleyella furcata	Chamaeleonidae: *Furcifer pardalis*	*Cx. quinquefasciatus*[1], *An. stephensi*[1]	Madagascar	Bain 1969b
Foleyella candezei (*F. agamae*)	Chamaeleonidae: *Bradypodion fischeri*	*An. stephensi*[1], *Ae. aegypt*[1]	Tanzania	Bain 1970
Foleyella philistinae	Agamidae: *Agama stellio*	*Cx. pipiens molestus*[1]	Lebanbon	Schacher and Khali 1968
Macdonaldius sp.				
Macdonaldius innisfailensis (syn. *Saurofilaria innisfailensis*)	Agamidae: *Physignathus lesueurii*	*Cx. quinquefasciatus*[1]	Australia	Mackerras 1962
Thamugadia sp.				
Thamugadia ivaschkini	Gekkonidae	*Phlebotomus caucasicus*[2], *Phlebotomus papatasii*[2], *Sergentomyia arpaclensi*[2]	Southern CIS	Reznik 1982
Madathamugadia sp.				
Madathamugadia ineichi	Cordylidae: *P. microlepidotus melanotus*	*Phlebotomus dubosqui*[1]	South Africa	Bain *et al.* 1993
Madathamugadia wanjii	Phyllodactylidae: *Ptyodactylus hasselquistii*	*P. dubosqui*[1]		Bain *et al.* 1992
Saurositus sp.				
Saurositus agamae hamoni	Agamidae: *A. agama*	*An. stephensi*[1]	Republic of Upper Volta	Bain 1969a

[1] Dipterans involved in transmission through experimental infection assays.

[2] Dipterans that has accomplished one or more criteria for incrimination established by Barnett (1962); but has not been confirmed through experimental infection assays.

Some hematophagous Diptera such as frog-biting midges (*Corethrella* sp.) and mosquitoes (*Uranotaenia* sp.) use the vocalisations of frogs to orient themselves and thus manage to locate vertebrate hosts (Bernal and Pinto 2016, Bernal *et al.* 2006, Borkent and Belton 2006, Camp *et al.* 2018, Netherlands *et al.* 2020a, Toma *et al.* 2014). Blood parasites use this relationship to extend their range of infection (Bernal *et al.* 2006). Netherlands *et al.* (2020a) studying guttural toad (*Sclerophrys gutturalis*) found that males show a higher infection rate with filaria than females, probably due to males' sounds during courtship. Such gender difference in the prevalence of

Trypanosoma tungarae was also reported in *Engystomops pustulosus* since males are more likely to be bitten by *Corethrella* sp. when emitting vocalisations of mating (Bernal and Pinto 2016).

Hemoparasites transmitted by hematophagous arthropods

Despite the great morphological diversity of parasites found in herpetofauna, including some specific genera such as *Saurocytozoon*, *Fallisia,* and *Progarnia*, most molecular characterisation studies have focused on blood parasites of mammals and birds. Additionally, there exist other factors that limit our knowledge of these parasites. First, there are biases in the taxon sampling. The second most crucial difficulty in the molecular characterisation of these blood parasites is nucleated erythrocytes in the vertebrate hosts; since the parasite's DNA is diluted, the parasite amplification becomes a problematic task as looking for a needle in a haystack. It turns limits genomic studies as well so that there are currently few molecular markers available. Recently, barcode sequences from mitochondrial markers have been used to characterise malaria parasites in herpetofauna (Perkins 2014, Maia *et al.* 2016, Matta *et al.* 2018). There is no such barcode sequence for identifying Adeloriniid parasites to this date.

The lack of genetic information about species of *Saurocytozoon*, *Garnia, Fallisia* is a significant obstacle to having a clear understanding of the phylogenetic relationship in Haemosporida order. This scenario is even worse for Adeloriniid blood parasites. For that reason, the taxonomic filiation is problematic and highly variable depending on the new molecular information added. In this review, we will follow the taxonomy proposed by Adl *et al.* (2019), while for Nematodes we will take into account the classification of Netherlands *et al.* (2020b) and Anderson (2000).

State of the art of hemoparasites transmitted by Diptera

Phylum Apicomplexa

Order Haemosporidia

Haemosporida **life cycle overview**

The haemosporidian are heteroxenous, which means that an invertebrate host (hematophagous vector) and a vertebrate host are involved to complete the life cycle. In the insects occurs the sexual or sporogonic phase that culminates with the infective forms called sporozoites. The merogonic or schizogonic phase occurs in the vertebrate host, releasing schizonts (*Plasmodium*, *Garnia*) and macro and microgametocytes (*Haemocystidum*, *Plasmodium*, *Garnia*, *Saurocytozoon*) in peripheral blood.

The insects ingest the macro and microgametocyte sexual forms. They transform into microgametes (male sexual forms) that exflagellate and fertilise the macrogametes (female sexual forms), forming zygotes. These will transform into ookinetes, which are mobile and invade the gut's epithelial cells, where they encyst and originate oocysts that harbour sporozoites. Then, hundreds of sporozoites are released into the hemocoel and migrate to the salivary glands. These infective forms are inoculated into the vertebrate when the blood-sucking insect has a meal (Valkiūnas 2005, Telford 2009).

Plasmodium Marchiafava and Celli, 1885

The family Plasmodiidae groups three genera: *Hepatocystis,* which infects mammals; *Polychromophilus* and *Nycteria*, which infects bats; and *Plasmodium,* reported infecting mammals, birds, and reptiles. It is important to note that no *Plasmodium* presence has been reported in amphibians to date, while about 100 species of this genus are estimated to infect reptiles. The first species described in reptiles was reported in 1909 concomitantly by Wenyon, Aragão, and Neiva in lizards from Sudan and Brazil, respectively (Aragão and Neiva 1909, Wenyon 1909). Garnham (1966) proposed three subgenera within *Plasmodium* based on purely morphological elements: those with large meronts are associated with *Sauramoeba*; small meronts with *Carinamoeba* and *Ophidiella*, which groups the *Plasmodium* species infecting snakes. Later, Telford (1988) proposed four more subgenera: *Paraplasmodium, Asiamoeba, Lacertamoeba*, and *Garnia*. To date, the phylogenetic reconstructions carried out with these last subgenera do not show a clear relationship with the morphological classification (González 2019).

Concerning the subgenus *Garnia*, the taxonomic classification continues to be controversial; some authors propose *Garnia* as a separate family (Lainson *et al.* 1971, Telford 1973). In this context Garnidae groups *Garnia, Fallisia*, and *Progarnia*. Unlike *Plasmodium* or *Haemoproteus* these genera are characterised by erythrocyte merogony and the absence of hemozoin granules. Some species of *Garnia* have been reported in birds and reptiles. In the Neotropics (Brazil, Haiti, Panama, and Venezuela), 11 species of *Garnia* have been described infecting lizards; *Fallisia* has been reported infecting only thrombocytes and leukocytes (Lainson *et al.* 1971, 1974, Valkiūnas 2005), and *Progarnia* that has been reported infecting crocodiles. However, further experimental infection of *Garnia* species in *Ameiva ameiva* demonstrated that the presence of hemozoin granules is variable, being invalid as a taxonomic feature to support Garnidae. There are no known vectors implicated in the transmission of *Garnia* and *Progrania* parasites.

Some studies analyse the impact of malaria infection in lizards, e.g. *Plasmodium mexicanum* can affect the social behaviour of *Sceloporus occidentalis* males due to the reduction of the testicular parenchyma and the reduction of the fat deposit resulting in loss of the ability to resist when escaping, but not affecting the explosive speed (Schall 1982). Schall and Sarni (1987) demonstrated a thermoregulation alteration in the same lizard species since the lizards spend a greater amount of time resting. On the other side, *Plasmodium floridense* and *Plasmodium azurophilum* alter the competitiveness between lizard species of the genus *Anolis* that coexist in the same habitat (Schall and Vogt 1993).

Parasites belonging to the genus *Plasmodium* have their sexual phase in mosquitoes and asexual stages in the vertebrate host. Meronts and gamonts can be seen in erythrocytes (mature and immature), thrombocytes, and leukocytes (Lainson *et al.* 1974).

Aragão and Neiva (1909) carried out the first assay to find a possible vector. They tried without success an experimental infection with *Culex confirmatus* and *Aedes taeniorhynchus,* seeking to feed on *Diploglossus fasciatus*, commonly known as snake lizard, infected with *Plasmodium diploglossi.* Later, Thompson (1946) explored an animal model to study antimalarials, studying lizards infected with *P. mexicanum* and *P. floridense* and found that both quinine and atebrin reduce the parasitaemia in lizards.

Surprisingly, a species other than mosquito was the first vector identified for a *Plasmodium* in reptiles; it is a sandfly *Lutzomyia vexator* (now *Micropygomyia* (*Coquillettimyia*) *vexator)*, the vector of *P. mexicanum* (Ayala 1971a, Klein *et al.* 1987). Using the lizard *Sceloporus occidentalis* (California fence swifts), infected with *P. mexicanum* as a food source for phlebotomine sandflies, they managed to demonstrate that 7-10 days post-infection, the insects developed oocysts and hundreds of free sporozoites in the hemocele and the salivary glands; it indicates that the transmission occurs when the insect feed. It subsequently infected 22 young lizards with sporozoites suspended in Ringer's solution, and an infection developed in three of them (Ayala 1971a). However, it is not the rule that phlebotomine sandflies function as vectors of *Plasmodium*, even in the reptiles themselves, since subsequent experiments with large numbers of these insects analysing *Plasmodium balli* or *Plasmodium fairchildi* were negative (Kimsey 1992). Ayala (1970) described the sporogonic development of *P. mexicanum* in *L. vexator* (now *M.* (*Coquillettimyia*) *vexator*) and *Lutzomyia stewarti* (now *Micropygomyia* (*Coquillettimyia*) *stewarti*) using wild sandflies captured in an area where the parasite was not present. He allows sandflies to take a blood meal on fence lizards, *S. occidentalis,* with high parasitemia. Engorged insects were kept at 24-26 °C in darkness and 75% of humidity. Ookinetes were observed 10-18 hours after feeding; they encysted and formed the oocysts in the sandfly's stomach. Sporozoites were found in flies' hemocoel and salivary glands 11 days after the infected blood meal.

Schall (2000) analysed if the proportion of gametocytes of *P. mexicanum* might influence the ability to infect *M.C. vexator*. He found a positive relationship between the proportion of microgametocytes and the infection rate of this species. Regarding the cycle in the vector Schall, (2000), as previously found Fialho and Schall, (1995) found that after nine days at 26 °C, it is possible to find oocysts in the sandflies. Another vector is *Culex* (*Melanoconion*) *erraticus*, which was experimentally implicated as a vector of *P. floridense* on *Anolis carolinensis* (Carolina anoles) (Kline *et al.* 1987). Oocysts appear in the insect two weeks after post-infection. Five anoles, infected either by a *Cx. erraticus* bite or intraperitoneal injection of sporozoites, developed infection 18-40 days post-infection. Given the great diversity of reptiles and *Plasmodium* species found, it is highly probable that other blood-sucking arthropods such as ticks or mites could be involved as vectors (Telford, 1994).

Saurocytozoon Lainson and Shaw, 1969

Saurocytozoon was first classified within the *Plasmodium* genus (Lainson and Shaw 1969), but later Ayala (1977) suggested that these parasites are related with *Leucocytozoon,* because they share morphological characteristics, and both seem to infect white blood cells. Lainson *et al.* (1974) proposed to separate *Leucocytozoon* and *Saurocytozon* based on the morphology of sporozoites. The first parasite, *Saurocytozoon tupinambi* was described from the Brazilian lizard *Tupinambis nigropunctatus*. Landau *et al.* (1973) studied whether sporogony occurred in *Cx. pipiens, Culex fatigans, Aedes aegypti*, and *Anopheles stephensi* infected with *Saurocytozoon tupinambi* fed from their vertebrate host *Tupinambis tequixin*. Only *Cx. pipiens* developed oocysts that reached their maximum size around day nine post-infection. Despite this, any sporozoites could be detected in salivary glands (Landau *et al.* 1973; Telford 1983).

Haemocystidium Castellani and Willey, 1904

Haemoproteidae has two genera *Haemoproteus* and *Haemocystidium.* The taxonomic classification of *Haemocystidium* has been controversial throughout history. This genus has changed to *Plasmodium* (Wenyon 1915) and *Haemoproteus* (Levine 1988, Wenyon 1926). Currently, *Haemoproteus* and *Haemocystidium* are classified as different genera, based on the absence of formation of 'pseudocytometers' in the meronts (located in the organs) (Telford 1996) and by molecular markers Pineda-Catalán *et al.* (2013). These authors suggest *Haemocystidium* should be split into two subgenera *Haemocystidium* (*Haemocystidium*) that infect vertebrate hosts of the order squamates, and *Haemocystidium* (*Simondia*), which infect chelonians (Figure 2) (Maia 2015, Perkins 2014, Telford 1996). To date there are 16 species identified (González 2019).

The tabanid *Chrysops callidus* is the only confirmed vector of *Haemocystidium metchikovi* (formerly *Haemoproteus* or *Simondia*) (De Giusti *et al.* 1973). The researchers found sporozoites in salivary glands of *C. callidus* insects captured in Oakland County, Michigan. The sporozoites were inoculated into the peritoneal cavity of laboratory-raised *Chrysemys picta,* and the researchers obtained gametocytes compatible with *H. metchnikov* at 30-32 days post-infection.

Order Coccidia*, Suborder* Adeleorina

Adeleorina parasites include different genus transmitted by Dipterans (mosquitoes, sandflies, tsetse flies, and biting midges), mites, leeches, ticks, kissing bugs, among others, using different mechanisms of transmission. Then it is challenging to make an overview of the life cycle traits for all these genera. Thereby, in this section, life cycles will be exposed in detail for each parasite genus.

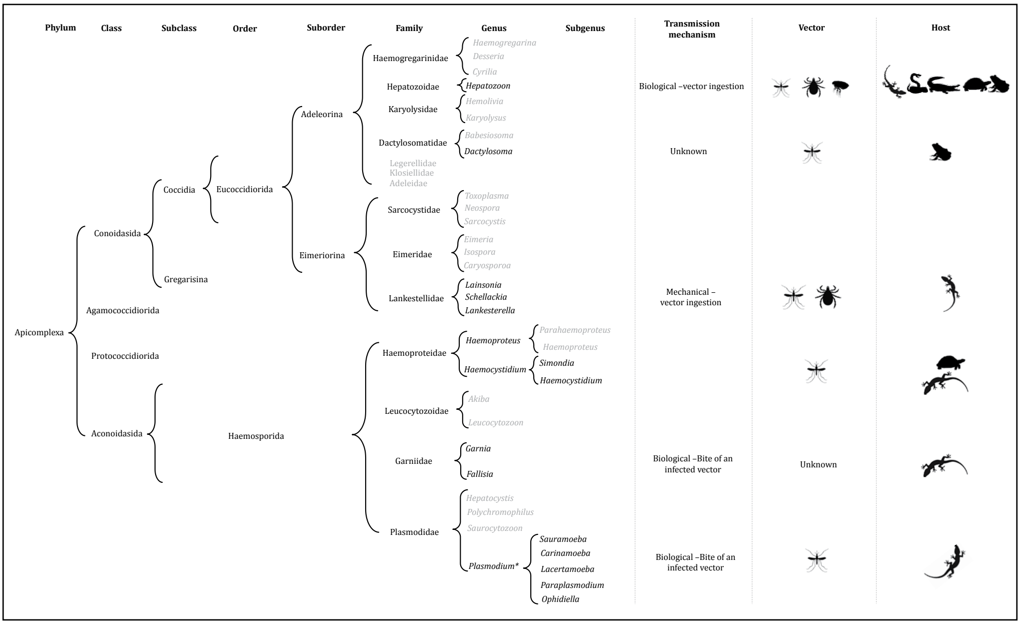

Figure 2. Taxonomic classification of Apicomplexa parasites according to Adl et al. *(2019), the arthropods involved in the transmission and the hosts in which such parasites have been reported. Genus/subgenus names in grey correspond to parasites not transmitted by dipterans or have not been reported infecting herpetofauna. Hosts: – turtles, – amphibians, – crocodiles, – snakes, – lizards. Vectors: – fleas, – ticks, – mosquitoes, – leeches.*

Hepatozoon Miller, 1908

Hepatozoon parasites were initially described as intracellular blood parasites of rats, transmitted by mites (Miller 1908). Nowadays, this genus of apicomplexans has been described in reptiles (Ball 1967, Telford 1984), birds (Hoare 1924), amphibians (Desser *et al.* 1995, Stebbins 1904), and even fishes from which it is believed could be paratenic hosts (Pereira *et al.* 2014) (an intermediate host that harbours an immature form of the parasite. Within the paratenic host, parasites do not undergo developmental stages or replicate but usually remain quiescent, then might not be necessary to complete the pathogen's life cycle (Porta 2014).

As well as they have many vertebrate hosts, different blood-sucking arthropods have been implicated in their transmission: fleas, mites, ticks, dipterans (e.g. mosquitoes, sandflies, tsetse flies), and reduviid bugs (Hemiptera) (Smith 1996). Although in the laboratory, transmission might be achieved by the bite of an infected insect (Ferguson *et al.* 2013), in nature, a vertebrate host gets infected with *Hepatozoon* through two mechanisms: ingestion of infected prey, either an infected insect or a vertebrate (Landau *et al.* 1972), and vertical transmission (Kauffman *et al.* 2017). *Hepatozoon* sporogonic life cycle begins when the mosquito feeds on an infected host (Figure 1), which blood contains mature gamonts that differentiate in macro and microgamonts. Such structures associate with syzygy for posterior fertilisation between the gametes differentiated from macro and microgamonts. After, a zygote that will give rise to an oocyst is formed. Within the oocyst, the sporoblasts and after the sporocysts appear (Smith 1996). In most life cycle characterisation studies, free sporocysts or sporozoites in salivary glands were not evident (e.g. Smith *et al.* 1994).

Vertebrate hosts may acquire the blood parasites by ingesting an infected invertebrate or an intermediate host. Sporozoites migrate to vertebrates' internal organs (liver, lungs, brain, and others) in the first case. The tropism for each organ depends on the parasite species, e.g. in *Hepatozoon catesbianae* (Desser *et al.* 1995), meronts are in the liver, while for *Hepatozoon aegypti* (Bashtar *et al.* 1984), the parasites develop in the lungs. After developing meronts (schizonts), merozoites are released to the bloodstream, where red blood cells are infected. In the second case, an intermediate host feeds on an infected invertebrate becomes a carrier of an infective stage by the production of cysts and the different stages of development of the schizogonic cycle of the parasite. Then, the vertebrate host that feeds on these intermediate hosts harbouring infective stages becomes infected (e.g. *Hepatozoon domerguei* or *Hepatozoon sipedon*, Landau *et al.* 1972, Smith *et al.* 1994).

Desser *et al.* (1995) fed *Cx. territans* on infected specimens of *Rana (Lithobates) catesbeianus.* Insects were maintained in captivity for more than 14 days until the sporogony was completed. It begins with the formation of the gametes, which occurs between the second-and third-day post-feeding (dpi). Fertilisation gives rise to the formation of a uninucleated zygote and a young oocyst within the first week. At 12 dpi, sporoblasts were developed, and finally, a mature sporocyst containing four sporozoites was observed.

The sporogonic life cycle of *Hepatozoon* species in herpetofauna shows variations in duration and places where the phase occurred. Experimental infections of *Hepatozoon gracilis* in the lizard *Mabuya quinquetaeniata* (Bashtar *et al.* 1987) revealed a shorter sporogonic life cycle (nine days until oocyst rupture), in contrast to the same process of *Hepatozoon catesbianae* in *Cx. territans* took 16 days (Desser *et al.* 1995). Moreover, *H. gracilis* the life cycle developments occur in the hemocoel instead of the Malpighian tubules as in *H. catesbianae.* Chronic infection by *Hepatozoon* sp. can negatively affect some reptiles' fitness. For example, in water-pythons (*Liasis duscus*) detrimental effects were reported on their reproductive and nutritional status, which finally ended up affecting individuals' survival. It has also been reported that individuals with high parasitaemia had slow growth (Madsen *et al.* 2005). Like many other microorganisms that develop inside an arthropod vector (Hardy *et al.* 1983), *Hepatozoon* parasites must overcome the immune response of the insect (Christensen *et al.* 2005), which affects the number of mature sporocysts negatively (Hardy *et al.* 1983).

Cx. pipiens has reduced fecundity caused by *Hepatozoon,* mainly when the infection is acquired from a heavily infected vertebrate host (Galal, 2010). Furthermore, changes in feeding behaviour could be related to the drainage of insects' energy and nutrients by the parasite (Ferguson *et al.* 2013). However, given the mechanism of transmission where the vector is predated, low mobility of insects either by the effects of the illness and/or natural processes of senescence; may favor the transmission (Ferguson *et al.* 2013).

Studies with *Hepatozoon clamatae* using *Cx. territans* revealed high mortality of insects infected in the laboratory (Harkness *et al.* 2010). Further to the deleterious effects of feeding on a heavily infected host, high mortalities have been recorded in non-natural vectors, which usually do not feed in the vertebrate hosts that bear the parasite; since the blood content may result not easily digestible, which in turn result in a fatal outcome (Paperna and Lainson 2004).

It is noteworthy that, although some species of *Hepatozoon* can be transmitted by mosquitoes and sandflies that have a recognised association with reptiles and amphibians, like *Cx. territans* or *Phlebotomus vexator* (now *Micropygomyia* (*Coquillettimyia*) *vexator)* (Bartlett-Healy *et al.* 2009; Shannon, 1914), there are parasite species transmitted by mosquitoes that are not usual feeders on herpetofauna. In fact, of about ten species of which a dipteran vector is known; three are transmitted by *Culex quinquefasciatus,* and another three are transmitted by *Cx. pipiens* which usually feeds on birds and in fewer cases on mammals (Apperson *et al.* 2004; Garcia-Rejon *et al.* 2010; Gomes *et al.* 2013; Hamer *et al.* 2008, 2009). Also, at least two are transmitted by highly anthropophilic vectors such as *Aedes* (*Stegomyia*) *aegypti* (Scott and Takken 2012, Telford *et al.* 2008). Such low specificity of vectors by their hosts may decrease by a dilution effect (Keesing *et al.* 2006, 2010) since the infectious bites may be given to a refractory or dead-end host.

Seasonality, along with other physical variables as landscape configuration, may affect the distribution of insects, generating variations of abundance and richness (Abella-Medrano *et al.* 2015, Chaves *et al.* 2011), which in turn may influence the vectorial capacity of each species.

Hepatozoon can be vertically transmitted (Kauffman *et al.* 2017), then assuring the parasite persistence along the year, since its transmission does not depend on vector availability (Antonovics *et al.* 2017); using this transmission mode, a closest host-parasite relationship may take place. Host-parasite cophylogenetic levels are highly influenced by the way of transmission and the dispersal ability (Santiago-Alarcon *et al.* 2014).

However, it is worth noting that the primary transmission mode of *Hepatozoon* is an indirect horizontal way in which the parasite uses vectors to be disseminated from host to host, as other Apicomplexa (Telford 2009). The Haemosporidian parasites, for example, uses Culicidae, Simuliidae, and other dipterans to be transmitted (Santiago-Alarcon *et al.* 2012, Valkiūnas 2005). Such dipterans show low host specificity and a high dispersal capacity, making it highly possible the host shifts, which is reflected in the low cophylogenetic signal (Lauron *et al.* 2015, Ricklefs *et al.* 2004, Santiago-Alarcon *et al.* 2014).

Despite *Hepatozoon* being transmitted by different arthropod species, some vagile than others, the transmission is achieved after ingesting low host-specific vectors or infected hosts (Smith 1996). Such a mechanism increases the dispersal ability. The host range relies on the infection success to a greater extent on the immune system of the vertebrate host than on the vector characteristics. This is reflected in the tangled phylogenetic relationships of these parasites, which do not correspond to the taxonomic classification of their vertebrate hosts (Tomé *et al.* 2014).

***Dactylosoma* Labbé, 1894**

In spite of the fact that *Dactylosoma* was described at the end of the nineteenth century, the knowledge about its taxonomy, distribution, transmission mode and vectors has been gathered in the recent fifty years (Manwell 1964). The genus was created to describe parasites of 'cold-blooded' vertebrates (fishes and anurans – Barta *et al.* 1987, Hoare 1930) that produces more than four merozoites when the erythrocytic merogony takes place (Barta 1991).

As these parasites are associated with aquatic or semi-aquatic vertebrates, leeches were the first candidates for transmitting them. The most closely related genus *Babesiosoma* is transmitted to their anuran hosts by the bite of an infected leech *Batracobdella picta* (Barta and Desser, 1989). Related studies registered the development of oocysts of *Dactylosoma ranarum*, in the midgut of an experimental leech. Further reports of Barta (1991) reveal that the life cycle was followed from the formation of a polysporoblastic oocyst to the formation of about 30 sporozoites per oocyst in the leech *Desserobdella picta*. However, authors could not determine the sporozoites' fate and failed to demonstrate the transmission to an uninfected host (Barta 1991). Additional studies performed to determine the role of other leeches failed to associate *Hemiclepsis margmata* on the transmission of *D. ranarum* (Boulard *et al.* 1982).

Further attempts to incriminate *Culicoides nubeculatus* as definitive hosts of these parasites did not render positive results (Boulard *et al*. 1982). However, a recent study performed by Netherlands *et al.* (2020a) managed to follow and document the sporogonic cycle of *Dactylosoma kermiti* in two *Uranotenia* species, *Uranotenia mashonaensis,* and *Uranotenia montana*. Mosquitoes were collected on the field and maintained alive until the appearance of the infective phase, which led them to gather more information about the parasite development than those obtained by Barta and Desser (1989). Then, in these experiments, parasite syzygy was observed one day post-ingestion (dpi). After the formation of an ookinete and a further oocyst were observed. Mature oocysts give rise to the formation of sporozoites which were observed free in the gut or the hemocoel at six dpi. None of such sporozoites was observed in the salivary glands, and their fate after being released from the oocyst is unknown.

On the other hand, meront-like structures containing ten merozoites appear to develop while the sporogonic cycle occurs. These merozoites are similar to those observed during the second merogony in the vertebrate. Their presence in the insect and the absence of sporozoites in salivary glands suggest a mechanism of infection through consumption of the infected vector-like in *Hepatozoon* parasites. However, according to the authors' observations, sporozoites lack the resistance structures typical of parasites whose transmission occurs by ingestion, leaving the infection mechanism an open question (Netherlands *et al.* 2020a).

Order Coccidia, Suborder Eimeriorina

Schellackia **Reichenow, 1919**

Schellackia parasites are placed in the Lankesterellidae along with *Lankesteralla* and *Lainsonia*. Little is known about the transmission of *Lankesterella* from which leeches have been considered as suspected hosts (Nöller 1912). There is no information available for *Lainsonia*, further than an experimental transmission carried out in the laboratory using the blood of an infected *Tupinambis nigropunctatus* injected intraperitoneally to another host from the same species (Landau *et al.* 1974).

Instead of this, arthropods such as mites and dipterans have been associated with the life cycle of *Schellackia* (Bristovetzky and Paperna 1990, Lainson *et al.* 1976). However, parasites do not undergo developmental changes within these invertebrates, making them act as mere transporters of a quiescent phase of the parasite (Klein *et al.* 1988).

Vertebrate hosts acquire the infection through the ingestion of an infected insect, sandflies (*L. vexator*), and mosquitoes (*Cx. erraticus, Culex molestus*) (Klein *et al.* 1988, Paperna 1993). The asexual division was dated by Lainson *et al.* (1976) from the 23rd day post-infection, where schizonts and some micro and macrogametocytes were evidenced. After 30 days of infection, some mature gametocytes are observed along with tissue oocysts in sub-epithelial tissue. Mature oocysts were reported at 45 days post infection.

On the other hand, Bristovetzky and Paperna (1990) followed the life cycle of *Schellackia agamae* in the lizard *Agama stellio*. Like other species of *Schellackia*, developmental phases occur in the small intestine of the vertebrate host. At five dpi, they observed young meronts, each containing more than five merozoites, and they completed their development seven dpi. After, 11 dpi, micro, and macrogamonts appeared and were observed until 14 dpi. The development of these structures overlaps the appearance and development of zygotes in the epithelial cells near the basal membrane. Transformation to oocysts begins at 14 dpi and may take up to four days to observe mature oocysts. After the sporozoites are released from the intestinal cells, they can be found in peripheral blood 25 dpi. After ingestion, sporozoites emerge from blood cells and penetrate the gut cells developing in quiescent forms which are consumed by the vertebrate hosts when they eat the invertebrate host.

Phylum **Euglenozoa**

Order Trypanosomatida

Trypanosoma **Gruby, 1843**

The Kinetoplastida class contains the Trypanosomatidae family, which currently encloses 19 genera, including *Trypanosoma* and *Leishmania* (Figure 3) (Kaufer *et al.* 2017). Trypanosomiasis is an infection caused by extracellular flagellated hemoparasites of the genus *Trypanosoma*, which infect mammals, birds, fish, reptiles, and amphibians. This genus has been reported worldwide except Antarctica. However, the most extensive records came from the tropical and subtropical regions (Telford 1984, 2009). Transmission involves an invertebrate hematophagous vector; among the main ones are ticks, leeches, sandflies, bed bugs, flies, lice, and fleas. In herpetofauna,

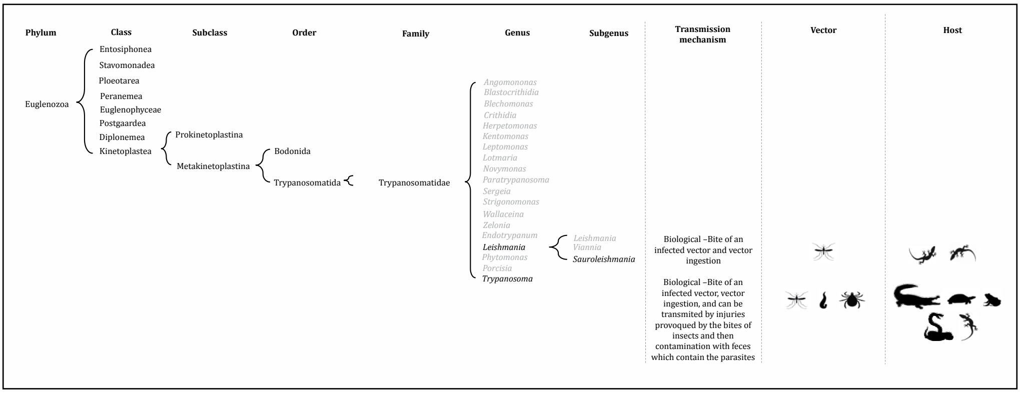

Figure 3. Taxonomic classification of Euglenozoa parasites according to Adl et al. *(2019), the arthropods involved in the transmission and the hosts in which such parasites have been reported. Genus/subgenus names in grey correspond to parasites that have not been reported infecting herpetofauna. Hosts: – turtles, – amphibians, – crocodiles, – snakes, – lizards. Vectors: – fleas, – ticks, – mosquitoes, – leeches.*

the main vectors involved are sandflies (Phlebotominae) and other Diptera, such as tsetse flies (*Glossina* sp.) (Telford 1984, 2009).

Depending on the *Trypanosoma* species and the vector implied, two different infective forms can be distinguished. Trypanosomes that infect herptile species with terrestrial habits have dipterans as vectors, and they become infected by ingesting trypomastigotes present in the blood. Trypomastigotes then transform into promastigotes and subsequently into sphaeromastigotes in the gut of the dipteran. Gradually the sphaeromastigotes will change into epimastigotes (infective stage), which migrate to the salivary glands. Therefore, their transmission then occurs by bite (Telford 1984, 2009). An exception to this life cycle occurs in the tsetse flies, where the trypanosome development does not produce epimastigotes as an infective stage but instead has a life cycle similar to that occurring within leeches (Hoare 1929), i.e. leeches are the primary vectors for aquatic herptiles. In a blood meal, the leeches ingest trypomastigotes, which transform into epimastigotes and multiply by binary fission to reach the infective stage, the metacyclic trypanosomes. These stages are in the terminal portion of the vector's intestine. When the vector bites and defecates, the metacyclic trypanosomes migrate towards the wound made by the vector's bites. The vector can also pass through the vertebrate's oral mucosa and enter the bloodstream, transforming into trypomastigotes (Telford 1984, 2009).

The relationship between reptile trypanosomes and dipteran vectors could have occurred more than 100 million years ago. Fossil studies carried out by Poinar Jr. (2008) confirm this theory since he found in a female *Leptoconops nosopheris*, fossilised in Lower Cretaceous Burmese amber, flagellate forms in the intestine, salivary glands, and salivary secretions. Observing these forms in detail in the digestive tract, he found structures compatible with *Trypanosoma* sp. He described these organisms as *Paleotrypanosoma burmanicus*, which constitutes the first fossil record of this genus of parasites. (Poinar Jr. 2008). Furthermore, the first report of dipteran capable of being infected with a reptilian *Trypanosoma* species dates from 1906. By this time, the presence of *Trypanosoma grayi* was evidenced within tsetse flies (*Glossina palpalis*) (Novy, 1906). However, the complete life cycle and transmission mode were only understood until 1929 by Hoare. *T. grayi* is found infecting Nile crocodiles (*Crocodylus niloticus*) and the transmission occurs by ingestion of the vector (*G. palpalis*) rather than a bite transmission (Hoare, 1929). The development of *Trypanosoma* in *G. palpalis* is similar to that described in leeches. As crocodiles maintain their jaws open while resting, tsetse flies easily get into their mouths. Then, such hosts may acquire the infection through the mucous membranes either when flies defecate the parasites or when the crocodile closes the jaws, crushing and subsequently ingesting crushed flies. Once in the crocodile bloodstream, parasites turn to the trypomastigote stage (Hoare 1929, 1931a,b).

In India the development of *Trypanosoma phlebotomi* was studied, using sand flies (*Sergentomyia babu shortti*) as a possible vector. Within the insect, the trypomastigotes migrate to the intestine and acquire spherical shape which, after 24 hours post ingestion (dpi), grew and formed cysts that housed proliferating amastigotes. later, at the fifth day, epimastigotes were present in the midgut and posterior intestine, and about the 8-9-day post-infection, the trypanosomes colonised the entire midgut, hindgut, rectum, and Malpighi's tubules (Shortt and Swaminath 1931). In nature, *T. phlebotomi* has been found in common geckos (*Hemidactylus frenatus*), and the authors suggest that under natural conditions, the transmission of the intermediate vector to the gecko occurs through ingestion of an infected sandfly (Shortt and Swaminath 1931).

Venipuncture sites in reptiles and small amphibians are challenging, in general, blood is obtained by cardiac puncture and could involve the sacrifice of animals. Currently, blood sampling techniques suggest keeping the specimen alive and favouring animal welfare (Giuseppe *et al.* 2017), so Adler *et al.* (1935) allowed *Phlebotomus parroti* (*Sergentomyia minuta*) and *Phlebotomus papatasii* (negative for *Trypanosoma* sp.) to feed on geckos (*Tarentola mauritanica*), to find parasitic forms in the dipterans and infer the diagnosis of reptiles to avoid sacrificing them. Individuals of *S. minuta* at three or four days after feeding showed flagellated forms in the oesophagus, even so, the authors do not report infection in the proboscis or the large intestine of the fly. For that reason, the authors hypothesised that the infection could occur by ingestion of the vector since a gecko was infected by swallowing one of these sand flies. However, they also did not rule out that the infection was caused by the bite of the vector within *P. parroti* and reported a mixed infection by *Trypanosoma platydactyli* and *Leishmania* (*Sauroleishmania*) *tarentolae* in the sandflies (Adler *et al.* 1935).

In western North America, *Trypanosoma gerrhonoti* and *Trypanosoma scelopori* have been recorded infecting southern alligator lizards (*Gerrhonotus multicarinatus*). The natural vector known to date is the sandfly *Micropygomyia* (*Coquillettimyia*) *vexator* (formerly *Lutzomyia vexatrix occidentis*). The development within this species was similar for both *Trypanosoma* species (Ayala and McKay 1971; Ayala 1970 cit. pos. Telford 2009). In the dissections made 18 hours after the blood meal, epimastigotes, pyriform flagellates, and spheromastigotes were found in the stomach and after 64 hours dense condensations formed by long and thin epimastigotes were also observed in the foregut. Finally, the cardias was obstructed by epimastigotes. The authors stated that both parasite species occur in coinfection (Ayala and McKay 1971). Shortly after, *Lutzomyia vexatrix occidentis* (now *Micropygomyia* (*Coquillettimyia*) *vexator*) was identified as a natural vector of *Trypanosoma bufophlebotomi* present in Californian toads (*Bufo boreas*). During the study, sandflies were extracted from the natural environment and allowed to feed on toads with high parasitaemia. Then between one and two days after blood feeding, amastigotes were observed in the stomach, and parasite agglomerations were also observed around the semi-digested blood meal, which later multiplied by binary fission; on the third day of infection appeared hundreds of small flagellates in the stomach, which migrate to the hindgut (Ayala 1971b).

Trypanosoma thecadactyli was described in 1972 in blood smears taken from forest geckos (*Thecadactylus rapicaudus*) in Panama. In the same microhabitat where blood samples were taken from the geckos, wild sandflies of *Lutzomyia trinidadensis* (now *Micropygomyia* (*Sauromyia*) *trinidadensis*) were also captured. Since such flies were already infected with *T. thecadactyli*, they were considered a natural vector; and the transmission by ingestion of the vector was postulated as a mechanism of infection. The infective forms were found in the posterior intestine of the sandflies. Flagellates were observed in the midgut and hindgut for 14 days in the entire intestinal tract in the studied flies, developing into epimastigotes (Christensen and Telford 1972). Furthermore, the authors attempted to infect laboratory-reared *Culex pipiens quinquefasciatus* (= *Cx. quinquefasciatus*) although no development of *T. thecadactyli* was observed (Christensen and Telford 1972).

As for *Trypanosoma rotatorium*, the complete life cycle was documented by Bailey (1962) in *Ae. aegypti*, being the ingestion of infected mosquitoes as the transmission mechanism. However, experiments carried out with *Cx. territans* under laboratory conditions showed its potential as a vector of *Trypanosoma* species in nature (Bartlett-Healy *et al.* 2009). Indeed, *Cx. territans* prefers to feed on cold-blooded vertebrates, mainly amphibians (Crans 1970). In further studies, artificially reared *Cx. territans* were allowed to feed on infected clamitans frogs. The trypomastigotes observed in the midgut of the mosquito, transformed into spherical amastigotes between one and two hours after ingestion, then multiplying within three and 70 h post-ingestion. Later, at 85 h, it was possible to observe small spheromastigotes in the posterior intestine, and the infective stage (epimastigotes) developed around 100 hours postprandial in the hindgut. Nevertheless, there were not observed mature or developing parasites in salivary glands. On the other hand, the authors report that mosquito mortality was low (Desser *et al.* 1973).

Moreover, individuals of *Mabuya striata* collected in Ethiopia presented low parasitemia of infection by *Trypanosoma boueti*. Despite this, when the reptiles were taken to the laboratory, it was possible to infect individuals of the sandfly *Sergentomyia bedfordi* extracted from the natural environment (Addis Ababa, Ethiopia). Amastigotes were found in the midgut 48 hours after ingestion of blood; after 72 hours, were transformed into spheromastigotes and transformed in the midgut to epimastigote in few hours (Ashford *et al.* 1973). However, the course of a concurrent bacterial infection in a sandfly caused the trypanosomes to die. Additional studies reveal the presence of *T. boueti* in specimens of the sandfly mentioned above species collected in Sebeta (Ethiopia), so it can be considered natural vector candidates (Ashford *et al.* 1973).

Sauroleishmania Ranque, 1973

The genus *Leishmania* includes the subgenera *Leishmania*, *Viannia*, and *Sauroleishmania* (Fraga *et al.* 2010). In spite that most *Leishmania* species infect mammals, *Sauroleishmania* has a tropism for lizards. The phylogenetic hypothesis suggests that *Sauroleishmania* evolved from *Leishmania* (Croan *et al.* 1997), in agreement with the high identity (90%) between human *Leishmania* genomes and *L.* (*S.*) *tarentolae* (Raymond *et al.* 2012).

The first discoveries of *Sauroleishmania* parasites were made by Chatton and Blanc (1914) and Sergent *et al.* (1915). Wenyon (1920) described the first species, *Sauroleishmania tarentolae* infecting *Tarentola mauritanica*. *Sauroleishmania* spp. have several differences with *Leishmania,* such as: (a) the host cell's amastigote are monocyte, thrombocyte or erythrocyte; (b) in the vertebrate host it can be found both the amastigote and promastigote; (c) the host are lizards and snakes; (d) the possible vector in the old world are species of *Sergentomyia* (Diptera, Psychodidae);

(e) the place where the parasite develops in phlebotomines are hypopylarian or peripylarian; and (f) genetic differences (Killick-Kendrick *et al.* 1986).

Sauleishmania subgenus has 19 valid species, infecting lizards from the old world; only a species is reported for Martinique Island (Lozano-Sardaneta *et al.* 2018, Tichá 2019). Overall, *Leishmania* parasites from reptiles are non-pathogenic for warm-blooded organisms. However, the zoonotic potential has been documented for *Leishmania (S.) adleri,* reported as a cause of cutaneous disease in humans, but can be asymptomatic in wild animals (Coughlan *et al.* 2017).

Bravo-Barriga *et al.* 2016 detected for the first time DNA of *L. (Sauroleishmania*) *tarentolae*-like in *S. minuta,* a sandfly with herpetophilic feeding habits. Then, Novo *et al.* (2015) and Pombi *et al.* (2020) also detected *Sauroleishmania* in the same diptera species in Africa and Italy, respectively. Even recently, *S. minuta* has been associated as a putative alternative vector of *Leishmania infantum* and *Leishmania major* (Pombi *et al.* 2020). The above-mentioned has led to the proposal of the infection of *Sauroleishmania* in lizards as a feasible animal model to study *Leishmania* parasites (Taylor *et al.* 2010).

The life cycle of these parasites is digenetic. The potential vectors are phlebotomine sandflies of the genus *Sergentomyia*; the promastigotes appear to develop both in the anterior and posterior part of the guts in the insects for that reason, the infection can occur by ingestion or by bite (Killick-Kendrick *et al.* 1986, Telford 1984, Tichá 2019, Wilson and Southern 1979). In the vertebrate host, the parasites can be free in the bloodstream, or amastigotes can be inside monocytes and precursors, thrombocytes or erythrocytes but also found in the cloaca (Simpson and Holz 1988).

Recently Tichá (2019) evaluated *Sauroleishmania* development in sandflies of the genera *Sergentomyia* and *Phlebotomus*. The main results pointed to differential development of *Sauroleismania* species associated with the potential vector analysed, e.g. *L.* (*S.*) *adleri* developed in the posterior gut (hypopylarian region) of *Sergentomyia schwetzi, Phlebotomus papteasi* and *Phlebotomus sergenti*. However, such development changed to the midgut (peripylarian region) in *Phlebotomus duboscqi, Phlebotomus orientalis* and *Phlebotomus argentipes.*

In contrast with the other blood parasites for the diagnosis of *Leishmania* in reptiles, the culture is critical since the prevalence of these parasites in peripheral blood is low. Similar occurs with *Trypanosoma* spp. These parasites are susceptible to be diagnosed by molecular tools.

***Phylum* Nematoda**

Suborder Spirurina

Family *Onchocercidae* Leiper, 1911

Microfilariae are extracellular parasites described as the 'embryonic' stage (L3) of nematodes known as filariae that can be found circulating in all vertebrates' connective tissue and body cavities except fishes (Anderson 2000, Szczepaniak and Tomczuk 2011). These nematodes are grouped within the Order Spirurida because it is believed that they shared a common ancestor early in their evolution with other nematodes of this order (Figure 4). They belong to the Filarioidea superfamily, which contains two families: Filariidae, which mainly infects the connective tissue of mammals, and Onchocercidae, the largest family, whose species have been reported to parasitise mammals, birds, reptiles and amphibians (Anderson 2000, Szczepaniak and Tomczuk 2011).

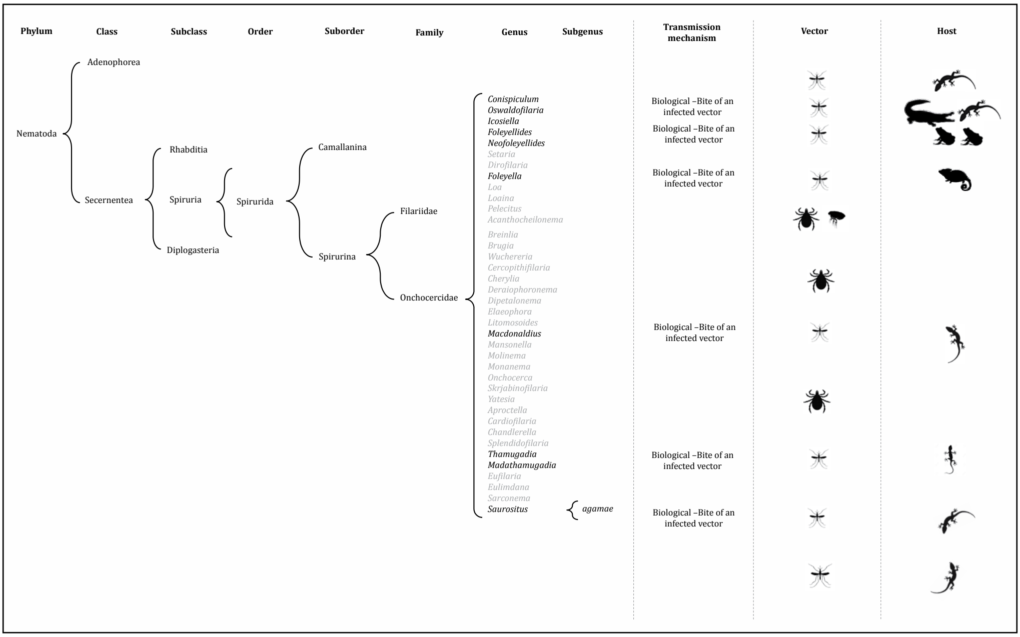

Figure 4. Taxonomic classification of Nematoda parasites according to Netherlands et al. *(2020b) and Anderson (2000), the arthropods involved in the transmission and the hosts in which such parasites have been reported. Genus/subgenus name in grey corresponds to parasites that have not been reported infecting herpetofauna. Hosts: – chameleons, – amphibians, – crocodiles, – lizards. Vectors: – fleas, – ticks, – mosquitoes.*

Filariae of Onchocercidae have been found mainly in lizards and snakes, although there are also reports in turtles, crocodiles, and amphibians. The pathogenicity of these nematodes depends on the filarial species, vertebrate health status, filaremia, and environmental conditions (Telford 1984). This family is composed of eight subfamilies; five of them (Oswaldofilariinae, Dirofilariinae, Onchocercinae, Splendidofilariinae, and Lemdaninae) have been reported infecting reptiles, being the genera *Foleyella*, *Macdonaldius*, *Oswaldofilari*a, and *Foleyellides* the most found in these hosts. Meanwhile, parasites of Icosiellinae and Waltonellinae have been recorded in amphibians (Anderson 2000).

Life cycle

Microfilariae begin their development in the adult female filaria's gravid uterus (within the vertebrate host). At this stage, they are surrounded by a yolk membrane, with a thin chitinous layer, like an egg. Depending on the species, females can be oviparous or ovoviviparous, giving birth to larvae (L1) known as microfilariae (Anderson 2000, Szczepaniak and Tomczuk 2011). In most species, the hatching of the 'egg' occurs in the uterus; thus, it is possible to observe them in the blood or skin of the vertebrate host without the sheath that covered them. On the contrary, some species of filariae retain these membranes; therefore, they circulate in the blood and skin of the vertebrate like an egg that will hatch only within the intermediate host (hematophagous vector) (Anderson 2000). Indeed, Szczepaniak and Tomczuk (2011) registered periodicity traits in which an increase of parasitaemia of the L1 microfilariae was observed synchronously with the peak of activity of the vector.

According to the filarial species, once ingested by the vector, they will have a tropism for different tissues such as fatty body, skeletal muscle (flight muscles), hemocoel, and Malpighi tubules. Inside the intermediate host, most of the species shorten and thicken, taking a 'sausage' shape to complete the larvae's cellular development (Anderson 2000). Subsequently, the larva experiences continuous growth until it undergoes the first molt (L2); then, a second molt (L2) occurs within the vector, lengthens a little more, and lastly, it molts for the third time to an L3 stage which is the infective stage for the vertebrate. Such mature stages move through the hemocoel to the head, where they reach the mouthparts of the vector (Kotcher 1941). The larvae's development process within the vector can take 10 to 30 days, under 19 and 32 °C for the stages (Szczepaniak and Tomczuk 2011). Then, the parasite migrates to the vertebrate's blood or skin when the arthropod feeds. The L3s come out of the mouthparts and direct towards the puncture wound made by the bite. Infectious larvae can measure approximately 0.60 to 5.00 mm in length, depending on the filarial species (Anderson, 2000). After the infection, the production of eggs may take from 41 days to nine months (Szczepaniak and Tomczuk 2011).

Inside the definitive host (vertebrate), larvae tend to go to the subcutaneous tissue, connective tissue (rarely tendons), mesenteries, under the serous membranes, peritoneal and pleural cavities, lungs, heart, blood vessels, and lymphatics. In amphibians, they can even migrate to the eyeball and eye sockets (Anderson 2000, Netherlands *et al.* 2020b). Transmission occurs through a hematophagous insect, generally mosquitoes (Culicidae) and sandflies (Phlebotominae), although ticks have also been involved (Telford 1984).

Vector research

When Manson (1878) performed studies on the transmission of *Wuchereria bancrofti*, the etiologic agent of human lymphatic filariasis, he assumed that the microfilariae had to escape from the vertebrate host to continue its cycle of development and ensure the survival of its species. Furthermore, he proposed that the stages he observed in human patients should theoretically continue to develop in a blood-feeding organism. Then, he discovered a series of morphological changes occurred until the infective stage in the abdomen of mosquitoes (he does not specify the species in the original publication). Subsequently, filariasis in reptiles was used as a research model to understand better the development and transmission of such illness in the vector, as both human and reptilian filariasis shared the same vector mosquito, *Cx. quinquefasciatus* (Menon *et al.* 1944).

Afterward, extensive research aimed to characterise the life cycle of such Nematoda parasites in different culicine species. Then *Cx. quinquefasciatus* has been involved in the transmission of five filaria species. Pandit *et al.* (1929), under experimental infection, demonstrated that the filaria *Conispiculum guindiensis* took 12 to 15 days to reach the infective stage (L3) in the mosquito proboscis (*Cx. quinquefasciatus*) when they were fed the blood of lizards (*Calotes versicolor*). Meanwhile, Causey (1939c) found that few larvae survive to reach the infective stage in the same vector species, probably due to a long incubation period. Besides, the studies revealed that in infections with high filaremia, the larvae's growth is retarded.

Moreover, Kotcher (1941) achieved an infection of *Cx. quinquefasciatus* after feeding on an infected frog (*Lithobates* sp.). During the experiments, the authors realised that not all ingested microfilariae managed to reach the infective stage since most were digested along with the blood 2 or 3 days after ingestion. Furthermore, they found 16 larvae of *Foleyellides brachyoptera* in an infectious state at 18 days post-ingestion in a mosquito, and 7 in an immature form (sausage-like shaped), which indicated that this species of filaria develops more easily within this species of intermediate host. They also reported that the life cycle of *F. brachyoptera*, takes three days more than that of *C. guindiensis*.

Cx. quinquefasciatus is a competent vector of *Macdonaldius innisfailensis*, parasitising *Physignathus lesueurii*, and *Oswaldofilaria spinosa* (Bain and Chabaud 1975, MacKerras 1962). Development *of M. innisfailensis* occurs in the mosquito's fatty body, and then infectious larvae were identified throughout the insect's body by 21 dpi (MacKerras 1962). Further experiments carried out by Brygoo (1963b) followed the detailed development of *Foleyella furcata* within the mosquito mentioned above species, finding that the L1 larvae appear around the 5th day; the L2 larvae after 13 days, and he found infective L3 larvae in the proboscis between 20-30 days post-ingestion at most, at temperatures of 12-20 °C. *Culex giganteus* has a limitation to transmit this filaria species, since larvae in an infective state were only observed in a single mosquito (Brygoo 1963b). Moreover, the southern house mosquito (*Cx. quinquefasciatus*) has been tested unsuccessfully to determine its role in transmitting *Piratuba varanicola*, a parasite of *Varunus* sp. and *Oswaldofilaria belemensis* (Bain and Chabaud 1975, MacKerras 1962).

Years later, *An. stephensi* was implicated as a vector of *F. furcata* under experimental conditions when insects were fed on infected *Furcifer verrucoso* from Madagascar (Bain 1969b). In Brisbane (Australia), Mackerras (1953) tested *Culex annulirostris,* a widespread native species, *Aedes notoscriptus*, *Aedes vigilax*, and *Anopheles annulipes* as vectors of *Oswaldofilaria chlamydosauri*. Although he could not incriminate *Aedes* and *Anopheles* species in the transmission of the parasite species, the development was successful in *Cx. annulirostris,* where the cycle takes about 23 days at 19-32 °C.

Other Culicidae species have been demonstrated to be susceptible to the infection caused by nematodes. Causey (1939c) showed that *Cx. pipiens* and *Ae. aegypti* could support the development of *F. brachyoptera*. Kotcher (1941) successfully managed to infect *Culex apicalis* with *F. brachyoptera*. In this species, infective stages were found in the insect's proboscis after eighteen days of development. Attempts performed by this author to experimentally infect frogs revealed that the infectious *F. brachyopter*a larvae require more than the presence of lymph as a stimulus to enter the vertebrate host.

Furthermore, *Ae. aegypti* and *Cx. pipiens* could transmit *Foleyellide*s sp. to frogs (*Lithobates pipiens* and *Lithobathes sphenocephalus*) under experimental infection, representing the first confirmed experimental vectors for amphibians. In nature, frogs present high filaremia (Causey 1939a).

Recently, Netherlands (2020b) described *Neofoleyellides boerewors* in the body cavity, subcutaneous tissue, and eyeball of guttural toad (*Sclerophrys gutturalis*). He reported on the development of *N. boerewors* in the fatty body of *U. mashonaensis* and *U. montana* in Sodwana, South Africa, with mosquitoes extracted from the natural environment. The larvae reached the infectious stage (L3) at 14-18 days in the thorax, head capsule, and proboscis of mosquitoes, supporting the theory that mosquitoes use the vocalisations of toads to locate them, leading to males' biases.

Diptera other than mosquitoes have also been implicated in the transmission of filariasis. Sandflies such as *Forcipomyia velox* and *S. silacea* allow the development of *I. neglecta* in the muscle tissue. The duration of larval development was between 22-26 days. The authors also tried to recreate the experimental infection in *Cx. apicalis* and *Cx. hortensis* because they fed on this frog species; however, no larval stage was observed (Desportes 1942, 1941). Other sandflies as *Phlebotomus caucasicus, P. papatasii, Sergentomyia arpaclensis,* and *Phlebotomus dubosqui,* allow the development of filarial species (*Thamugadia ivaschkini, Madathamugadia wanjii,* and *Madathamugadia ineichi*) present in geckos (Gekkonidae) from the old world (Bain *et al.* 1992, 1993, Reznik 1982). The infectious stage was evidenced in the dipteran head from the eighth to the seventeenth day postprandial (Bain *et al.* 1992, 1993). For *M. ineichi* infectious larvae were observed in the thorax of sandflies seven days after infection (Bain *et al.* 1993).

Microfilariae are the most harmful parasites for mosquitoes. Experimental studies have revealed a high mortality rate of such vectors within the first 48 hours after ingestion (Brygoo 1963b, Causey 1939a,b,c, MacKerras 1953, Pandit *et al.* 1929). Besides, the higher the concentration of ingested microfilariae, the more serious the infection and therefore the percentage of mosquito that dies is higher (Witenberg and Gerichter, 1944), due to the trauma caused to the intestinal wall caused by the migration of the larvae towards the mosquito hemocoel (Causey 1939a, MacKerras 1962, Pandit *et al.* 1929).

In mosquitoes infected with high charges of parasites, it is possible to find parasites in different vectors' bodies, even in the head (MacKerras 1953). A similar scenario has been observed in other parasite infections where vectors ingest high parasite loads along with the blood meal (Bukauskaitė *et al.* 2016). Mosquitoes show little activity after ingesting blood infected with a high load of microfilariae (these mosquitoes end up dying a few hours later) (Causey 1939a). In species, such as *Foleyella philistinae*, which invade the mosquito's fat body (*Cx. pipiens disturbus*), a slight initial reaction of the fat body has been evidenced. It was accompanied by increased density and granular appearance of the cytoplasm around the larva as larval development progressed (Schacher and Khalil 1968).

To date, the lack of information on relevant aspects in the ecology of the filariae transmission cycle in herpetofauna constitutes a challenge and many possibilities to develop research. It is necessary to continue researching to determine the filariae species, which parasitise amphibians and reptiles, and the description of new natural vectors to understand the infection's ecology. Furthermore, the filarial species' zoonotic potential that infects herpetofauna remains unknown. Future research is necessary to evaluate these aspects.

Zoonosis

With the current COVID-19 pandemic, the magnitude of anthropic activities' impact on wild animal populations is evident. This pressure could promote the appearance of new zoonotic diseases for human and animal health (Decaro *et al.* 2020). The zoonoses expert committee of the World Health Organization (WHO) in 1951 defined zoonoses as 'diseases and infections that are naturally transmitted between vertebrate animals and man.' The classification of zoonotic diseases has changed over time, and different ways of dividing them have been proposed. In the beginning, zoonotic diseases were grouped according to the direction of transmission, i.e. anthropozoonotic diseases (from animals to humans) and zooanthroponotic diseases (from humans to animals). They are also classified according to the etiological agent, such as viral, bacterial, parasitic, fungal zoonoses, or even by unconventional agents such as prions (Chomel 2009). Currently the most widely used classification is the primary epidemiological one, that is, according to the infection cycle, in this concept there are four types of zoonoses: (1) Orthozoonoses: also known as direct zoonoses, they are those where a vertebrate host can transmit the infection to another susceptible, either by direct contact or by contact with a fomite, this type of zoonosis can occur within the same species of vertebrates; (2) Cyclozoonosis: these are those that need more than one species of vertebrate, but do not use an invertebrate host to complete the cycle of the pathogen; (3) metazoonosis or ferrozoonosis: contrary to the previous group, these zoonoses need a vertebrate and an invertebrate host, for example diseases transmitted by vectors, within the invertebrate, the incubation period of the pathogen occurs, and it can merely multiply (cyclopropagative transmission) or develop (developmental transmission) before it infects a vertebrate host; and (4) Saprozoonosis: here in addition to having a vertebrate host, they require an inanimate site (food, soil, plants, and environment) to complete development (Chomel 2009).

The herpetofauna harbour a wide variety of organisms, such as protozoa, helminths, pentastomids, and bacteria; some of these can represent a risk to human health (Mendoza-Roldan *et al.* 2020). Overall, bacteria of the genus *Salmonella* sp. are the most common pathogens reported that cause zoonotic diseases (Corrente *et al.* 2017). Much of the studies focus on bacteria's zoonotic potential, leaving aside parasites and many vector-borne parasites. Usually, most pathogens that cause zoonoses use reptiles as intermediate or paratenic hosts rather than definitive hosts (Mitchell 2011). Currently, the demand for reptiles as exotic pets has increased, with an estimated 21% of the live animal trade being reptiles (Engler *et al.* 2007, Mendoza-Roldan *et al.* 2020). In several cultures, reptiles represent a fundamental food source, and raw flesh is used in traditional medicine. Therefore, there is a risk of people being exposed to zoonotic agents harboured by reptiles (Mendoza-Roldan *et al.* 2020).

Metazoonoses or zoonoses transmitted by vectors associated with herpetofauna are still mostly unknown. However, studies on the transmission of *Leishmania* sp. showed that herpetophilic sandflies (*S. minuta*) not only transmit non-pathogenic species such as *Leishmania tarentolae* but can also carry species that cause Leishmaniasis in humans, such as *L. infantum* and *L. major* (Bravo-Barriga *et al.* 2016, Campino *et al.* 2013, Latrofa *et al.* 2018, Pereira *et al.* 2017). Besides, *Leishmania (S.) adleri* was reported infecting mammals also. In a mummified human in Brazil, *L. tarentolae* was identified in the bone marrow; this is a non-pathogenic parasite species for humans originally described as infecting geckos from the old world. Until now, there is no clear evidence of the degree of replication of these parasites in human macrophages (Novo *et al.* 2015). This triggers alarms since the investigations raise the hypothesis that lizards and these sandflies could participate in the epidemiological cycle of Leishmaniasis in humans together with the vectors.

The pet trade mainly influences the introduction of wild or exotic animals to new geographical areas; the translocation, introduction, or reintroduction of animals always represents a risk to human and environmental health. It is because animals carry pathogens and introduce them to new ecosystems with subsequent harmful effects on wildlife/domestic populations and changing ecological balance in an area (Kock *et al.* 2010).

When there is no prior monitoring and diagnosis of the herpetofauna's health status at the time of entry to a particular country or region, the parasites they harbour easily cross epidemiological barriers, managing to find means for their spread (de Sá and Melo, 2016). For example, in the early 1960s a filaria *(Macdonaldius oschei*) was described in necrotic tissues of pythons (*Python bivittatus* and *Malayopython reticulatus*) kept in the Stuttgart zoological garden (Germany). However, in the same zoo, the rainbow boas (*Epicrates cenchria*) also had a chronic infection with *M. oschei* but showed no clinical signs (Frank, 1962), necrosis in the skin and muscles of pythons was attributed to occlusion of the arteries caused by microfilariae of *M. oschei*, which may eventually lead to the accidental death of the host. Apparently, the pythons contracted the infection due to the introduction of New World snakes to the zoo, which also brought *M. oschei* with them in their blood; they also carried the vector of this filaria; presumably, a tick (*Ornithodoros talaje*) that was capable of infecting pythons (Frank 1964). Although this report involves a tick as a vector, the same situation could occur with dipteran vectors when introduced to new geographic areas.

Introduce, reintroduce, or translate wild fauna to a particular site must follow biosecurity protocols (Cunningham 1996). Rigorous compliance with these preventive measures increases the release's success and reduces the movement of parasites between different habitats (Cunningham 1996, Germano and Bishop 2009, Viggers *et al.* 1993).

Conclusions

Identifying species of blood parasites in wildlife is time-consuming and requires trained personnel. Therefore, the research groups, schools of Biology, and Veterinary, Ecology, must promote human resources interested and trained to carry out these tasks. On the other hand, the study and characterisation of blood parasites in wildlife require a multidisciplinary team in charge of molecular and morphological diagnosis and take care of biological collections. The gap in the knowledge of blood parasites in herpetofauna is evident; in this way, significant efforts should be addressed (1) to increase the diversity of taxa sampled; (2) genomic studies that allows designing new molecular markers with potential use as barcode; (3) to increase the knowledge of complete life cycles; that implies knowing the vector's development; (4) establishing animal models likely could help overcome some of these challenges.

To date, the lack of information on relevant aspects in the ecology of the filariae transmission cycle in herpetofauna constitutes a challenge and represents many possibilities to develop research. Furthermore, the filarial species' zoonotic potential infecting wildlife remains unknown. Future research is necessary to gain a better understanding of the ecology and dynamics of the transmission cycles of blood parasites that infect herpetofauna and their relationship with dipterans, as a tool for the conservation of reptiles and amphibians, given the current decline in these animal taxa and their susceptibility to various pathogens (Bower *et al.* 2019).

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4. Zoos and wildlife parks: a laboratory for the study of mosquito-borne wildlife diseases

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Abstract

Zoos and wildlife parks offer a variety of biotopes and habitats for all life stages of numerous mosquito species and are places of close encounters between mosquitoes, captive animals, wild native animals and human visitors. Since stock animals of modern zoos/wildlife parks are closely observed by their keepers and medically attended by veterinarians, disease agents transmitted to them by mosquitoes will come to attention as soon as they cause symptoms or the animals are routinely checked. In addition to giving medical care to the affected animal, zoo/wildlife park staff has then the possibility to induce epidemiological investigations. The latter should be done in cooperation with the higher veterinary authority or, in case of a zoonotic disease agent, with the responsible public health authority, or with both. Zoos/wildlife parks can thus be valuable sentinel stations for detecting local circulation and transmission of mosquito-borne disease agents and significantly contribute to public health surveillance. Sometimes, zoos/wildlife parks also offer the opportunity to study mosquito-borne infections or disease cases in susceptible non-natural hosts or in natural hosts kept under non-natural conditions or exhibiting non-natural behaviour. These situations allow important insights into disease etiology and pathology as well as into vector biology. With respect to the 'One Health' approach, zoos, wildlife parks and similar facilities, where exotic and non-adapted animals are exposed to locally occurring (native or imported) vector-borne pathogens, should gain much more attention regarding pathogen surveillance and public health issues.

Keywords: animal health, biosurveillance, pathogen transmission, public health, sentinel, vector

Introduction

Detecting rare, including emerging, endemic and zoonotic vector-borne pathogens in the field can be a search for the needle in the haystack. However, the knowledge on the presence and circulation of such pathogens in a given area will help raising awareness and facilitate or direct the design and implementation of measures to prevent or manage disease cases among animals and humans (Braks *et al.* 2011). Accordingly, disease and pathogen surveillance and research in wildlife is a major issue of the One Health approach (Sleeman *et al.* 2019).

For reasons of resources, vector-borne pathogen surveillance is rarely done comprehensively and proactively. It usually only occurs in response to noticeable events such as the emergence of local disease cases or infections, or the establishment of a new vector species. Surveillance may be performed on potential vectors with acquisition of these to be screened for pathogens (xenomonitoring) usually taking place by random trapping, and less often by collecting from human or animal baits (Silver 2008). Much more elaborate and delicate is the examination of animal hosts for infection, as these have to be arduously captured and sampled by invasive

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techniques. Moreover, such activities usually underly protection of animal acts and have to be formally permitted (Soulsbury *et al.* 2020). Both vector and vertebrate samples can be examined for the pathogens by microscopy (histology of body fluids or tissue samples), culturing (of body fluids) or biochemical and genetic methods (e.g. MALDI-TOF, PCR assays) (Reller *et al.* 2017). Additionally, vertebrates can be indirectly tested for infection by serological techniques (Kuleš *et al.* 2017) and, in some cases, directly by xenodiagnosis (e.g. Meiser and Schaub 2011, Mourya *et al.* 2007, Turk *et al.* 2018). However, large-scale programmes on vector-borne pathogen surveillance are rarely in place, and pertinent activities are often only performed in the framework of research projects.

A special situation exists in zoos and wildlife parks, where animals are generally under thorough observation and, in most cases, routinely and regularly examined for their health status (Carpenter *et al.* 2016). Certainly, this is particularly important for animals taken from the field or originating from unknown or dubious sources to supplement the animal stock in a zoo/wildlife park. In addition to being medically examined, they often have to pass a quarantine period before being released to their conspecifics in order not to introduce any external disease agents (Carpenter *et al.* 2016). However, nowadays animals are rarely taken from the field to be held captive in zoos. Most of them result from breeding programmes that aim to conserve species at risk of extinction or increase the genetic diversity of animals and therefore have already been born and bred in captivity, hence have been medically attended since birth (IUCN 2014, Mallinson 1995). Based on this, it can be assumed that animals are healthy when they enter a zoo and may acquire an infection only in the present zoo setting. This implies that vector-borne pathogens detected in zoo animals are generally not exotic but have a local source.

Haematophagous ectoparasites that may serve as vectors in zoos or wildlife parks are rarely stationary and permanent, since these have little chance of being introduced. Due to the impact they have on hosts, medical checks are routinely performed on introduced vertebrates, with their high detection capacity leading to the quick detection and elimination of the ectoparasites by pest control activities. In addition, stationary and permanent vectors have limited occasions to circulate pathogens, unless between vertebrates of the same flock or in the same enclosure. Vector-borne pathogens are therefore mainly introduced by mobile arthropod species difficult to control, with mosquitoes playing a crucial role (Adler *et al.* 2011).

Mosquitoes in zoos/wildlife parks

Mosquitoes as potential vectors of pathogens find excellent developmental and living conditions in zoos and wildlife parks (Heym *et al.* 2018). First, these provide numerous and different kinds of breeding sites (Tuten 2011). Zoos and wildlife parks usually contain a lot of small and medium-sized water places of any kind in order to offer appropriate and pleasant settings for both captive animals and visitors. Containers used as drinking troughs are probably unproblematic as mosquito production sources since the water is replaced every few days at least, not permitting aquatic mosquito stages to complete their development. However, other man-made constructions, be they ornamental or necessarily linked to buildings and animal shelters, such as rainwater gutters, gullies and cesspits, may collect water and serve as persistent mosquito breeding habitats. Second, zoos and wildlife parks are usually heavily vegetated providing shadowy places where adult mosquitoes rest and plenty of sources to acquire nectar and sweet plant juices. Finally, potential blood hosts are available in considerable numbers: captive vertebrates, free-roaming native birds, rodents and other small vertebrates, and human visitors (Vezzani 2007).

Mosquito eggs, larvae and pupae can be imported into zoos/wildlife parks by plants and gardening equipment, while adults may enter from outside, e.g. urban or forested areas, depending on their flight radius. For example, the average flying distances of *Culex pipiens* and *Aedes vexans*, two of the most common and widely distributed mosquito species in Europe, are 400-500 hundred and a good hundred metres, respectively (Verdonschot and Besse-Lototskaya 2014).

Thus, mosquitoes are omnipresent in zoos and might easily transmit pathogens from wild native animals to captive animals and between visitors and zoo animals.

Mosquito-borne infections in zoos/wildlife parks

Except for avian malaria, publications on mosquito-borne infections in zoos and wildlife parks are few, suggesting either a limited number of cases or underreporting in the scientific literature. As the task of zoo and wildlife park staffs has no scientific priority, the latter is more likely. Of the pertinent literature published, most articles pertain to infections with viruses and avian malaria parasites.

Viral infections

Several reports exist on mosquito-borne viral disease incidents in vertebrates or on mosquitoes infected with viruses, in particular West Nile virus (WNV), from zoos and wildlife parks. While published studies on infections in vertebrates mainly refer to seroprevalences, those on mosquitoes predominantly describe genetic pathogen detection approaches.

Expectedly, anti-WNV antibodies cannot only be found in vertebrates believed to be susceptible to WNV infections (i.e. may develop disease symptoms), such as certain groups of birds and horses, but in others having come into contact with infectious mosquitoes as well, e.g various mammal or reptile species (see below). However, also symptomatic WNV infections were observed in exotic animal species. Zoo birds fell ill or were tested seropositive in considerable numbers, e.g. in Kansas and New York City (USA), Córdoba (Spain) and Tabasco (Mexico) (Cano-Terriza *et al.* 2015, D'Agostino and Isaza 2004, Hidalgo-Martínez *et al.* 2008, Ludwig *et al.* 2002). In the Bronx Zoo, New York, captive vertebrates were found seropositive for infection during the 1999 WNV outbreak, with 34% of tested birds and 8% of tested mammals positive (Ludwig *et al.* 2002). Comparison with stored sera provided no evidence for WNV infection before 1999 and demonstrated that the virus did not originate from a captive bird introduced into the zoo stock.

In one zoo in Tabasco (Mexico) about a third, each, of all tested birds (numerous species) and mammals (several species including antelope and big cats) were seropositive, while six of seven tested crocodiles (86%) had anti-WNV bodies. One pool of *Culex quinquefasciatus* was found WNV-positive by PCR (Hidalgo-Martínez *et al.* 2008).

Most remarkable were the cases of symptomatic WNV infections in a polar bear in the Toronto Zoo in Canada in 2006, and in an orca in the SeaWorld park in Texas, USA, in 2007. The bear was euthanised after displaying paraparesis and a poor general condition. It was tested seropositive for WNV after symptoms had been recognised, and WNV could be cultured and detected by PCR in spleen samples taken post mortem (Dutton *et al.* 2009). Infection in orcas is particularly noteworthy as these would probably hardly become infected in nature where exposition to mosquitoes is difficult to imagine: in their natural marine habitat, orcas are mostly not close enough to the shore and do not spend enough time at the water surface for mosquitoes to bite. In addition, mosquitoes keep away from turbulent waters as these pose a threat to their life. Notwithstanding, infections with WNV as well as with eastern, western and Venezuelan encephalitis viruses have serologically been shown to occur in free-ranging Atlantic bottlenose dolphins, although no disease symptoms were registered (Schaefer *et al.* 2009). The orca in the Texas SeaWorld died suddenly without obvious signs of illness but autopsy showed lesions of the central nervous system typical for WNV infection. Also, WNV was genetically confirmed in brain tissue (St. Leger *et al.* 2011).

In captivity, orcas spend most of their time just floating motionless at the smooth water surface of their pools, in a surrounding where mosquitoes may easily approach and feed (Jett and Ventre 2012). Jett and Ventre (2012) see further risk factors of orcas to suffer from mosquito-borne viral diseases in their generally poor health condition in captivity, resulting from (1) higher UV radiation and epidermal lesions following sunburn due to a lot of time spent at the water surface in marine parks in the southern USA as compared to their natural occurrence at more northern latitudes, and (2) ill dentition due to biting concrete and metal structures in their spatially limited pools.

After the repeated occurrence of WNV cases among captive birds in the Berlin Wildlife Park in 2018 and 2019 (Ziegler *et al.* 2019, 2020), Kampen *et al.* (2020) screened mosquitoes collected in the park in autumn 2019 and found seven WNV-positive *Cx. pipiens* complex pools including the two biotypes *pipiens* and *molestus*.

WNV is also a health threat to humans and horses, although being dead-end hosts for the virus (Chancey *et al.* 2015). Most infections remain undetected and pass by without any symptoms but some 20% of the infected individuals develop unspecific flu-like symptoms. While most of these quickly recover, the virus becomes neuroinvasive in 1% of the patients and may lead to severe impairment of the central nervous system including death (Rossi *et al.* 2010).

A similar situation is given with St. Louis encephalitis virus (SLEV) which is believed to have killed an orca already in 1990 in the Florida SeaWorld (Buck *et al.* 1993): most patients will recover spontaneously, some will display mild symptoms and only few, mainly elder ones, will develop encephalitis. For the latter, the case fatality rate can reach 20% (Reisen 2003). The orca in Florida died after presenting with reduced appetite and lethargy. Diagnosis was based on neutralisation tests and immunofluorescence staining, supported by virus-induced cytopathic effect in inoculated cell cultures and electron microscopy of culture cells (Buck *et al.* 1993).

Neutralising antibodies against SLEV, as well as against Zika virus (ZIKV) and dengue virus (DENV) serotypes 2, 3 and 4, were also found in capuchin monkeys in the Recife Zoo, Pernambuco, Brazil (De Oliveira-Filho *et al.* 2018). Both ZIKV and DENV can lead to severe disease symptoms in humans. ZIKV gained global attention during the large epidemic in South America in 2015 and 2016, with numerous children born with microcephaly (Hills *et al.* 2017). In adults, ZIKV infection may be associated with Guillain-Barré syndrome (Silva *et al.* 2020). DENV infection may either remain asymptomatic or have a mild or severe disease course. The mild outcome is characterised by flu-like symptoms, while the severe one may include dengue hemorrhagic fever and dengue shock syndrome. The risk of developing severe dengue increases with a second infection of a different virus serotype (Silva *et al.* 2020).

In Connecticut, USA, 14 African penguins (*Spheniscus demersus*) out of a flock of 22 held at a public aquarium were diagnosed with eastern equine encephalitis (EEE) in 2003 (Tuttle *et al.* 2005). This was the first time, clinical signs of EEE virus (EEEV) infection had been described in penguins.

Diagnosis was based on hemagglutinin inhibition and virus neutralisation tests after development of substantial neurological signs in 13 of the penguins. The same number of penguins survived due to intensive supportive care while one juvenile penguin had to be euthanised since clinical symptoms did not improve. Although very rare in canines, too, two Mexican gray wolf pups died of EEEV infection in Binder Zoo Park, Michigan, USA, in 2019 (Binder Park Zoo 2019). EEEV is a primary virus of horses. In humans, flu-like symptoms may occur, but severe disease cases have been recorded with fatality rates of up to 35% (Kumar *et al.* 2018).

Usutu virus (USUV) has become a common and widespread mosquito-borne virus in Europe. It emerged in Austria in the early 2000s (Weissenböck *et al.* 2002), although it was later shown to have been present in Italy already in 1996 (Weissenböck *et al.* 2013). Since that time, avian USUV infection and mortality were recorded across many European countries (Vilibic-Cavlek *et al.* 2020), with several cases described from zoological gardens or private aviaries in Switzerland, Germany, Austria, Spain and the Netherlands (Becker *et al.* 2012, Buchebner *et al.* 2013, Cano-Terriza *et al.* 2015, Rijks *et al.* 2016, Steinmetz *et al.* 2011, Ziegler *et al.* 2016). USUV is a virus of birds, and its zoonotic potential is still under discussion. Few symptomatic cases have to date been reported in humans, mainly splenectomised patients (Cavrini *et al.* 2009, Pecorari *et al.* 2009). However, rare neuroinvasive cases have also been described from people without any pre-existing adverse conditions (Nagy *et al.* 2019, Simonin *et al.* 2018, Vilibic-Cavlek *et al.* 2014).

Sindbis virus (SINV), mainly transmitted by *Cx. pipiens, Culex torrentium, Aedes cinereus* and *Aedes excrucians* (Lundström 1994), can cause sporadic illness that is rarely connected to a fatal outcome in birds and a flu-like disease in humans which sometimes leads to chronic arthritis (Hubálek 2008). This virus was detected in a pool consisting of *Cx. pipiens* complex mosquitoes collected in the Berlin Wildlife Park (Heym *et al.* 2019).

Protozoan infections

Compared to other vector-borne infections in zoos and wildlife parks, a relatively large body of literature exists on cases and outbreaks of avian malaria and malaria-like disease which are caused by protozoan parasites of the genera *Plasmodium*, *Leucocytozoon* and *Haemoproteus*. Only species of the genus *Plasmodium*, which are ubiquitous and common, are believed to be transmitted by mosquitoes, while *Leucocytozoon* parasites are thought to be mainly transmitted by black flies (Simuliidae) and *Haemoproteus* by biting midges (Ceratopogonidae) and keds (Hippoboscidae), respectively (Service 2001). While these parasites generally do not do appreciable harm to co-evolved and adapted birds, they may be life-threatening to, and cause mass mortality in, bird species not adapted to the infectious agent (Bennett *et al.* 1993, Valkiūnas 2005). In the early 19th century, the *Cx. quinquefasciatus* mosquito was introduced into Hawaii by humans. This species, a competent vector of avian plasmodial protozoans, enabled the transmission of *Plasmodium relictum* from introduced to native birds and almost caused the extinction of Hawaiian honeycreepers (a passerine subfamily) due to avian malaria (Van Riper *et al.* 1986, Warner 1968). A high diversity of genetic lineages of avian malaria parasites suggests the existence of numerous undescribed species (Valkiūnas and Iezhova 2018).

In zoos, penguins are particularly susceptible to avian malaria parasites, and disease outbreaks have been documented from all over the world (e.g. Baltimore Zoo, USA: Stoskopf and Beier 1979; Farmland Zoo, Korea: Bak and Park 1984; São Paulo Zoo, Brazil: Bueno *et al.* 2010; Hai Park Zoo, Israel: Lublin *et al.* 2018). Certain species of penguins present particular high sensitivities to this parasites, probably caused by the fact that they have little or no contact to mosquitoes and mosquito-borne pathogens in their natural surroundings, resulting in a naive immune system (Jones and Shellam 1999, Jovani *et al.* 2001). The high mortality rates of up to 50-80% in captivity due to avian malaria have prompted many zoos to systematically test their penguins for plasmodia and to administer antimalarial drugs prophylactically (Grilo *et al.* 2016).

However, avian malaria infections caused by plasmodia have also been reported from captive bird species other than penguins, e.g. pheasants (Murata *et al.* 2008), yellowheads (Alley *et al.* 2008), Egyptian goose (Fernandes-Chagas *et al.* 2013), flamingos (Thurber *et al.* 2014) and various species of cranes (Jia *et al.* 2018).

As opposed to the considerable number of articles on avian malaria in zoos and bird holdings, hardly any studies have been published on mosquito vectors in these settings. In a zoological garden in Kanagawa (Japan) Eijiri *et al.* (2009) found plasmodial DNA in *Lutzia vorax* and *Cx. pipiens* group mosquitoes which they specified as *Cx. pipiens pallens* in a subsequent study (Eijiri *et al.* 2011). Heym *et al.* (2019) demonstrated the DNA of *Plasmodium* sp. in *Cx. pipiens* biotype *pipiens* as well as *Haematoproteus-* and *Leucocytozoon*-DNA in undefined specimens of the *Cx. pipiens* complex in the Berlin Wildlife Park and the Eberswalde Zoo, Germany. In the Barcelona Zoo (Spain), Martínez-de la Puente *et al.* (2020) found DNA of various *Plasmodium* strains in *Cx. pipiens* s.l. and of a *Haemoproteus* strain in *Culiseta longiareolata*. In Bioparco Zoological Garden and Zoomarine Park in the Lazio region, Central Italy, Iurescia *et al.* (2021) identified *Plasmodium matutinum*-DNA in both deceased African black-footed penguins and in *Cx. pipiens* s.l., and *Plasmodium vaughani*-DNA in *Cx. pipiens* s.l. only. In the São Paulo Zoo, Brazil, De Oliveira-Guimarães *et al.* (2021) demonstrated *Plasmodium nucleophilum*-DNA in *Aedes albopictus, Aedes scapularus, Culex acharistus* and *Mansonia indubitans*, in addition to non-specified plasmodial DNA in indeterminable *Culex* species. Interestingly, *Haemoproteus*-DNA was found in head/thorax samples of an *Ae. scapularis* female and three *Mansonia* sp. females, suggesting presence of metacyclic stages of this haemosporidial genus, which is considered to be non-culicid-borne, in the mosquitoes' salivary glands. At Aquarium Marinepoia Nihonkai, Niigata, Japan, Inumaru *et al.* (2021) identified two *Plasmodium* lineages in both mosquitoes fed on penguins (*Cx. pipiens* group, *Culex tritaeniorhynchus*) and in penguins, whereas *Plasmodium cathemerium* was only detected in mosquitoes, suggesting that captive and wild birds have their own specific roles in avian malaria.

Filarial infections

Filarial worms may produce disease in all groups of vertebrates, and several species are zoonotic. For example, culicids transmit species of the genera *Dirofilaria* (pathogenic to carnivores and humans), *Setaria* (pathogenic to ruminants and equids) and *Cardiofilaria* (pathogenic to birds) (Mehlhorn 2016).

The heartworm *Dirofilaria immitis* is a parasite of caniforms, transmitted by mosquito species of various genera. In addition to humans, it can occasionally be found in feliforms, such as in the case of a snow leopard in the Kobe Municipal Oji Zoo, Japan (Murata *et al.* 2003). The leopard died of pancreatic carcinoma, and the filariae were incidentally detected in the right cardiac ventriculus during necropsy. During the leopard's lifetime, they had not caused any obvious clinical signs. More extraordinary is the symptomatic infection of a Humboldt penguin by *D. immitis* in Akita Municipal Omoriyama Zoo, Japan, assumed to be causative for the death of the penguin (Sano *et al.* 2005). The filariae were found post mortem in the right atrium of the heart and in the connective tissue of the lung. In both, leopard and penguin, filarial species diagnosis was carried out using morphological and genetic tools.

Contribution of zoos/wildlife parks to mosquito-borne disease research

Mosquito ecology

Ecological and behavioural characteristics of mosquito vectors contribute to presence and abundance as well as pathogen transmission. Zoos and wildlife parks offer excellent surroundings to analyse mosquito blood meals that provide conclusions about host preferences and feeding behaviour (Heym *et al.* 2018, Martínez-de la Puente *et al.* 2020, Schönenberger *et al.* 2016, Tuten *et al.* 2012). The identification of breeding and resting sites of specific mosquito species gives valuable information for exposure reduction in zoos or even zoo construction. Furthermore, flight distances can be accurately assessed for blood-fed mosquitoes by measuring distances between their collection sites and the enclosure of the vertebrate species fed upon (Greenberg *et al.* 2012, Heym *et al.* 2018, Martínez-de la Puente *et al.* 2020, Schönenberger *et al.* 2016, Tuten *et al.* 2012). *Ae. vexans*, for example, was captured at an average distance of 109.2 m (18 m minimum, 167 m maximum) away from its blood host in the Rio Grande Zoo, Albuquerque, New Mexico (Greenberg *et al.* 2012), while *Cx. pipiens* s.l. exhibited mean post-blood meal flight distances between 62 and 236 m, depending on the gonotrophic conditions (Sella stage), in Greenville and Riverbanks zoos, South Carolina (Tuten *et al.* 2012) and a mean post-blood feeding flight distance of 99 m (maximum 168.5 m) in Barcelona Zoo, Spain (Martínez-de la Puente *et al.* 2020).

In addition to blood of various zoo animal species found in the mosquitoes, human blood was detected in *Ae. albopictus, Aedes annulipes* s.l.*, Ae. cinereus, Aedes japonicus, Aedes punctipennis, Ae. vexans, Anopheles claviger, Anopheles maculipennis* s.l.*, Anopheles plumbeus, Culex erraticus, Culex hortensis* and *Cx. pipiens* s.l. (Greenberg *et al.* 2012, Heym *et al.* 2018, Martínez-de la Puente *et al.* 2020, Schönenberger *et al.* 2016, Tuten *et al.* 2012).

Deeper knowledge of all these aspects facilitates the preparation of risk assessments for both zoo animals and human citizens, as well as the design and implemention of targeted vector and disease management. A closer collaboration between the animal and public health sectors is therefore most desirable.

Biosurveillance

As routine surveillance of mosquito-borne pathogens in the field is exceptional, zoos and wildlife parks play a paramount role as epidemiological monitoring stations and significantly contribute to the detection of zoonoses and emerging infectious diseases. Domesticated and captive animals are kept close to humans, sharing the same or similar environments, but spend much more time outdoors, thereby displaying an increased exposure risk (Rabinowitz *et al.* 2013). Thus, the routine work of zoos and wildlife parks on attendance and treating of animals in the interest of conservation also significantly serves the public health sector (McNamara 2007). It can be assumed that infections in zoo animals will be detected relatively soon, either during routine examinations or after the onset of symptoms, and that they will be taken care of and followed up for their etiology. This includes re-examination of test material taken previously from the same animals which are commonly kept banked for some time. The animals are frequently observed and monitored, and in the case of death undergo complete necropsy and any additional disease testing to determine the cause of death (Binder Park Zoo 2020).

The contributions zoological institutions are able to make for public health and their relevance have been identified and analysed retrospectively for the case of WNV in the US (Pultorak *et al.*

2011). During the 1999 outbreak in New York, examinations of fresh and archived animal samples from the Bronx Zoo helped unveil the etiology and epidemiology of mysterious avian morbidity and mortality, identifying the cause as WNV infections (Ludwig *et al.* 2002). To this end, animals represent important sentinels for the surveillance of human health hazards in general (Neo and Tan 2017), with a particular role of zoo/wildlife park animals, in addition to exhibiting (exotic) biodiversity and serving for conservation purposes. Moreover, captive animals rarely live under natural conditions in zoos and wildlife parks, i.e. in different geographical areas and/or forced to display non-natural behaviour, and might be exposed to pathogens not occurring in their native distribution area and natural habitats. Hence, combinations of pathogen and host may turn up not found in nature. This may be detrimental to the individual vertebrate affected, but may significantly add to our knowledge on pathogen-host interactions, vector ecology, and disease pathology and epidemiology. In this context, it is to be kept in mind that haematophagous arthropods in which pathogens are detected by genetic techniques may not necessarily be vectors of the demonstrated pathogens. Most often, the whole bodies of arthropods are homogenised for examination, not differentiating between crucial body parts such as midgut and head/salivary glands. Only demonstrated experimental transmission or the detection of infectious pathogens in the salivary glands (viruses, protozoa) or head (filarial worms) after natural infection qualifies a mosquito to be called a biological vector.

Climate change

Zoos and wildlife parks can also contribute to infectious disease research with respect to climate change as they may have possibilities to simulate different climatic conditions and have available long-term historical data on animal health (Barbosa 2009). Of 118 transmissible diseases affecting animals in zoos and aquariums (Kaandorp 2004), 29, including several vector-borne ones, are considered likely to be impacted by climate change (Barbosa 2009). Therefore, under the auspices of the World Association of Zoos and Aquariums (WAZA), project MOSI was established, a zoo-based mosquito monitoring network aimed at assisting wildlife health management and contributing data on mosquito spatio-temporal distribution changes (Quintavalle Pastorino *et al.* 2015). In this programme, composition, abundance and acitivity profiles of mosquito species and their changes over time are to be determined in various European zoos. Moreover, mosquitoes will be examined for blood meal sources and pathogen presence. Assistance in risk evaluation and management of mosquito-transmitted pathogens in the zoo environment will be provided.

Concluding remarks and future directions

Although several mosquito species collected in zoos and wildlife parks have been found infected with mosquito-borne disease agents pathogenic to animals held captive, knowledge on their vector competence, vector capacity and actual vector role in these particular settings is scarce. Prevalence studies on wild animals foraging within the zoos or within flight distance of mosquito species occurring in zoos that may present infection sources, such as birds, might help better assess the infection risk for zoo animals and design prevention strategies. Measures to control mosquitoes and reduce exposition of zoo animals are mainly sanitary, i.e. consist of elimination, reduction and proper management of potential mosquito breeding places, be they natural or artificial (Derraik 2005). The regular application of larvicides (e.g. *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* toxins; Lacey 2007) to water bodies in and around the zoos and the use of insect meshes on cages with particularly endangered species and facility windows is worth considering. However, monitoring of both zoo/wildlife park animals and mosquitoes for pathogens is a most efficient early warning tool that allows to take targeted action in due time to prevent or reduce mosquito-borne disease agent transmission to both zoo animals and humans.

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Mosquito feeding behaviour

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5. Effect of host preferences of mosquitoes on disease transmission between wildlife and humans

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Abstract

Some disease agents are readily transmitted by mosquitoes from one host species to another host species, while others like *Plasmodium falciparum* are host specific. This is mainly determined by the susceptibility of the host and the vector competence of the mosquito for a certain disease agent. However, the mosquito has to bite the host to be able to transmit the disease agent, which is determined by host availability and host preference. Mosquito host preference drives the transmission of vector borne pathogens within and between species. Mosquitoes with an opportunistic feeding behaviour may be responsible for the transmission of pathogens between host species, while mosquitoes with specific preferences may facilitate the maintenance of pathogen cycles within a specific host species. The factors that drive host preference and therefore disease transmission from wildlife to humans are discussed. For Chikungunya, malaria, Zika and West Nile, and overview is given of the role of mosquito host preference on the transmission to and from wildlife. The tools to study the host preferences of mosquitoes in wildlife settings are limited and are often biased or difficult and expensive to organise. Therefore, the spillover of pathogens from wildlife to humans and vice versa by mosquito vectors is still poorly understood. Because deforestation and an increased human population will lead to closer contact between wildlife, humans and mosquitoes, monitoring at locations where spillover may occur will become more important.

Keywords: host finding, host selection, mosquito behaviour, pathogens, vector diseases, zoonosis

Host preference and disease transmission

Blood-sucking insects transmit numerous infectious disease agents that affect humans, livestock and wildlife (Marcondes *et al.* 2017). Several factors affect the transmission dynamics of vector-borne parasites that involve mosquitoes, parasites and vertebrate hosts. One of these factors is mosquito host feeding preference. While some mosquito species feed mainly on birds (ornithophilic species), others may primarily select mammals (mammophilic species, including humans characterised as anthropophilic species), or even amphibians or reptiles. However, many mosquito species are opportunistic, feeding on the most accessible host (Gutiérrez-López *et al.* 2020, Molaei *et al.* 2008, Muñoz *et al.* 2012, Takken and Verhulst 2013). The degree of host specificity may suffer variation depending on the mosquito species, or other factors, including host availability and abundance (Takken and Verhulst 2013). For example, Muñoz *et al.* (2012) analysed the blood meals of *Culex pipiens*, which is classified as an ornithophilic species, and it was shown to feed on a wide variety of vertebrate host species like birds and mammals, including humans. In contrast, *Aedes aegypti* and *Aedes albopictus* showed elevated preference to feed on humans (Sivan *et al.* 2015). In a particular mosquito species with a clear host preference, changes in their feeding behaviour could facilitate the transmission of pathogens between different vertebrate

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taxa. For example, the West Nile virus (WNV) circulates under natural conditions between wild birds transmitted by a wide range of vectors, but occasionally humans and horses act as incidental hosts of the virus causing serious outbreaks (Kilpatrick *et al.* 2006).

Mosquitoes are hematophagous and need blood proteins as essential nutrients for egg production. Selecting the proper vertebrate host for feeding is essential for mosquito's life history, as well as pathogen transmission. The host preference of mosquitoes is therefore an important determinant in the epidemiological dynamics of vector borne diseases.

Host selection versus host preference

In this book chapter we define host preference according to Takken and Verhulst (2013) as: 'The trait to preferentially select certain host species above others'. However, the host preferred by a mosquito may not be the host selected for a blood meal, as host selection will mainly depend on host availability. The preferred host may not be abundant or even absent in an area, or the host may have found ways to reduce contact with mosquitoes. Humans for example make use of bednets or house screening to limit contact with mosquitoes. Chimpanzees may build their nests higher in the canopy or at higher altitude to avoid mosquito bites (Koops *et al.* 2012, Krief *et al.* 2012). The method selected to determine host preference will bias the outcome and often reflects host selection rather than host preference. Collecting blood fed mosquitoes inside houses for example will lead to the collection of a high number of mosquitoes that fed on human blood, but outdoor catches will probably result in a higher proportion of animal blood meals. The methods to determine host preference in wildlife settings will be discussed later in this chapter. If host availability in the area of mosquito collection is taken into account, the differences between host selection and host preference may become clear. Jaleta *et al.* (2016) for example analysed blood meals from *Anopheles arabiensis* caught indoors and outdoors. Although they found that the selected hosts were different between the indoor and outdoor catches, the proportions did not reflect the host abundance, indicating a preference for certain hosts and avoidance of others.

What mediates host preference of mosquitoes

The choice to bite a particular host species by mosquitoes is determined by several cues, specifics to each host. These visual, chemical and thermal cues define the interaction between hosts (including humans) and vectors, affecting the transmission of pathogens.

Sensory cues

Mosquitoes use a combination of multiple sensory cues to detect, identify and select their hosts, including visual cues, concentration of carbon dioxide (CO_2), host size, temperature, moisture, host colour, and body odour (Lehane 2005). The integration of these sensory cues allows host identification. In addition, mosquitoes can also detect signals emitted by hosts that reflect the host´s health status, such as infection with a pathogen.

Mosquitoes present three organs in their head that constitute their peripheral olfactory system, which they use to locate hosts, oviposition sites and food (sugar secretions). These appendices are the palps, the antennae and the proboscis. The antenna contains hair like-structures called sensilla, also present in the palps and in the proboscis but in less proportion than in the antenna (Hill *et al.* 2009). These sensilla are responsible for the chemical, thermal and hygro-detection when their sensory neurons are activated by host´s emanations.

The most important cue for mosquitoes to find their host is CO_2 in exhaled breath. Approximately, 4-5% of human breath exists of CO_2 while in the atmosphere only the 0.04% is present (Gillies 1980). Mosquitoes can detect CO_2 from a long distance (>10 m) due to its highly volatility (Van Breugel *et al.* 2015). Fluctuating levels of CO_2 signify a nearby living vertebrate and is detected by the carbon dioxide-sensitive neurons present in palps (Grant *et al.* 1995). The host seeking response of mosquitoes is higher when both, odour and CO_2 are detected simultaneously instead of either stimuli alone (Tauxe *et al.* 2013), although the degree of attractiveness to CO_2 varies between species (Mboera and Takken 1997). The role of the concentration of CO_2 emitted by hosts is less important when mosquitoes present anthropophilic feeding preferences with the aim to distinguish specific humans volatiles from other vertebrate hosts (Spitzen *et al.* 2008).

The use of volatile chemical cues consists on the recognition of species-specific characteristics and even individual identification (Bruce *et al.* 2005). Olfactory receptor neurons situated across the sensilla on the antennae and palps perceive host volatiles. Mosquito response of different types of volatiles depends mostly on their host feeding preferences. Female *Culex quinquefasciatus* that feed on birds and mammals, including humans, have one of the most developed olfactory sensilla with five morphological types of sensilla on each antenna that use on the recognition of specifics odour blends coming from hosts (Hill *et al.* 2009). In contrast, four types of sensilla are present in the antenna of the anthropophilic *Anopheles gambiae* mosquitoes (Qiu *et al.* 2006). Differences in the number of sensilla, and therefore, in the quantity of receptors, could be involved in the mosquito selection among vertebrate hosts.

Animals emit large quantities of volatile organic compounds across their skin. Human odour is composed by a complex mixture of different compounds, with more than 400 compounds detected by gas chromatography coupled with mass spectrometry, including alcohols, aldehydes, carboxylic acids, ketones and sulphides (Dormont *et al.* 2013). Compounds like lactic acid, octenol or ammonia play a role in the attraction of mammophilic mosquitoes such as *An. gambiae* and *Ae. aegypti* (Schreck *et al.* 1982, Takken and Knols 1999). The community of microorganism present on the skin plays an important role in the production of these volatiles. The skin microbiota is involved in the degradation of the volatile and non-volatile compounds and produces metabolic by-products that alter the biochemical profile of an individual. When the richness, the relative abundance and the diversity of the bacterial community is variable between individuals, the compounds resulting from their degradation are different too, and therefore, the odour emitted by every single individual is different (Maraci *et al.* 2018, Takken and Verhulst 2017).

Birds produce an oily secretion in their uropygial gland that spreads over their plumage. Principal compounds of uropygial secretion are relatively similar to the compounds present in human odour, (Campagna *et al.* 2012), however, when the attraction of mosquitoes to the uropygial secretion was analysed, contradictory results were reported. While some studies showed the attraction of mosquitoes in traps supplemented by this secretion (Russell and Hunter 2005) others did not find significant differences in the response of *Cx. pipiens* to the secretion (Díez-Fernández *et al.* 2020). It is possible that microbiota present on the skin of birds degrades this uropygial secretion and modify, at least in part, birds body odour. Additionally, these chemical signals may provide particularly effective indicators about the individual's health and their infection status. This relation has important consequences in the disease dynamics, in particular, in the case of *Cx. pipiens* as one of the principal vectors of WNV, with birds as principal reservoirs and humans and horses as incidental hosts. If the odour of birds changes accordingly with the infection status and increase the mosquito attraction, thereby, incrementing the possibilities of the transmission of WNV to a new host. For the case of WNV this assumption is still poorly studied, however, in the case of the *Plasmodium*-infection in birds, mice and humans, the infection modifies the volatile profile of individuals increasing the attraction to mosquitoes.

In close ranges, the role of the body heat and the moisture produced by host may serve as useful cues to locate them, even with differences in temperature of 0.2 °C (Lehane 1991). This response of a mosquito to heat from a host increases when it is combined with other stimuli like odours (Spitzen *et al.* 2013) and vice versa the addition of heat and moisture to odour baits increases the capture rates of *An. gambiae* mosquitoes (Olanga *et al.* 2010). However, thermal- and hygrosenses in mosquitoes remains poorly understood and there is currently little evidence that it plays a role in the selection of one host species above another.

Another host morphological trait that mediates the mosquito selection behaviour is their colour. The contrast between the host and the environment, and the brightness may be particularly useful for host discrimination in combination with the shapes and size of the host. The peak of spectral sensitivity in *Ae. aegypti* has been determined in the ultraviolet and green wavelengths (Muir *et al.* 1992). Colours such as black, blue and red are more attractive to mosquitoes than white, so mosquitoes prefer dark areas to land on with low-intensity colours (Chambers *et al.* 2013). Contrary with these assumptions, Yan *et al.* (2017) found that *Cx. pipiens* were more attracted to light colours as light brown and green, yellow and white. Some mosquito species are diurnal while others are crepuscular/nocturnal which could be related to their attraction to different colours. Specific host body colouration could interfere in the mosquito host selection and therefore, in vector-host interactions and have implications in the transmission of infectious diseases between species.

It is commonly thought that the role of visual cues is triggered by the concentration of CO_2 emitted by the host and determines the flight direction. Animals with higher body mass have elevated rates of oxygen consumption associated with their metabolism, and therefore, exhale higher concentrations of CO_2. As a result, bigger hosts could be more attractive to mosquitoes. A study with *Culex erraticus* and *Culiseta melanura* suggested the influence of host size in mosquito searching behaviour by showing a positive relation between avian body mass and mosquito forage ratio (Estep *et al.* 2012). According to Yan *et al.* (2017), birds with longer tarsi attract more *Cx. pipiens* to feed, suggesting the importance of exposed skin in mosquito feeding process, especially when the length of tarsi is positively related to body mass (Green 2001). In this regard, it is also important to consider the anti-mosquito behaviour of the host. This behaviour is reduced in bigger-size animals with higher areas of skin exposure while smaller individuals may present an active defensive behaviour against mosquitoes (Mooring *et al.* 2000).

Genes and learning

Host preference of mosquitoes is determined, at least in part, by a genetic background. Infectious diseases have an impact on vertebrates, where the most well studied association of immune system are the Major Histocompatibility Complex (MHC). MHC is a multi-gene family that encodes more than one hundred proteins involved in the adaptive immune response (Klein *et al.* 2007). The MHC plays an important role in the susceptibility and resistance to diseases. Furthermore, MHC genes influence individual odours (Penn and Potts 1998), although is still unclear how this process occurs. Variation in the genes of MHC in mice favouring specific urinary odour composed of a mixture of volatiles carboxylic acids (Singer *et al.* 1997). The MHC may also influence human body odour (Savelev *et al.* 2008), and therefore, the attractiveness to mosquitoes. The anthropophilic *An. gambiae* mosquito showed a higher attraction to human skin emanations from individuals with the human leukocyte antigen gene *Cw*07*, which are involve in the regulation of the volatile composition (Verhulst *et al.* 2013). Additionally, Fernández-Grandon *et al.* (2015) evaluated the genetic basis of the attraction of *Ae. aegypti* with the body volatiles odour of pairs of identical and non-identical twins. Results demonstrated that mosquitoes are capable to distinguish genetic differences through the human odour profile, which was used in host selection.

It would be interesting delve into the mechanism underlying host discrimination. There is evidence that indicate mosquitoes may have the ability of learning, which is associated with their positive or negative experience in their previous blood-feeding episode (Chilaka *et al.* 2012). For example, Vinauger *et al.* (2014) examined under laboratory conditions the ability of *Ae. aegypti* to associate odorants with the opportunity to obtain blood meal. These results showed that mosquitoes forming memory depends on the success of previous feedings episodes (Vinauger *et al.* 2014). Hence, this ability has an epidemiological impact, facilitating the transmission of different pathogens between several hosts (Alonso and Schuck-Paim 2006).

Infection (of mosquito or host)

In studies on the evolution of parasite transmission it is important to integrate the relationship between the hosts, the vectors and the parasites. Parasites can affect the behaviour, appearance and physiology of their hosts to increase their transmission success (Poulin 2011). One of the most studied parasite manipulations are from the malaria parasites in their hosts and in their mosquito vectors. This parasite modulates the biting behaviour of their vectors, showing higher biting rates to obtain the same quantity of blood in *An. gambiae* infected with *Plasmodium berghei* sporozoites (infected forms of parasite) than uninfected ones (Koella *et al.* 2002). Similarly, *An. gambiae* infected with *P. falciparum* showed a higher attraction to human odours than uninfected mosquitoes (Smallegange *et al.* 2013). These changes in the mosquito attraction may be facilitated by the alteration in the olfactory system of vectors implemented by the infection to facilitate parasite transmission. When sporozoites of the rodent malaria parasite *P. berghei* are present in *An. gambiae,* the proteins spots in their head are altered and suffer a modification in their olfactory system (Lefèvre *et al.* 2007). In contrast, mosquito host-seeking behaviour has been attenuated when oocysts, the stage of the parasite that cannot be transmitted, maintain the mosquito in an inactive activity (Cator *et al.* 2013), decreasing the risk associated with feeding and so, trying to keep the mosquito alive to complete the parasite development.

Other pathogens, such as dengue virus that infect *Ae. aegypti* increase the feeding time of infected mosquitoes compared to uninfected ones, which increment the time for the transmission of virus and facilitate the host defensive behaviour with the interruption of feeding event but favouring to probe on an additional host (Camara *et al.* 2011, Platt *et al.* 1997).

To summarise, mosquitoes host-seeking behaviour is altered by infection of pathogens. This manipulative system plays a role in the feeding process and therefore it has implications in the dynamic of pathogen transmission.

Zoonotic transmission of important vector borne diseases

Chikungunya

Chikungunya virus (CHIKV) belongs to the genus *Alphavirus* of the *Togaviridae* family. Commonly, the virus circulates in a sylvatic cycle between non-human primates (NHPs) reservoir hosts and

Aedes species mosquitoes. CHIKV causes periodic and explosive outbreak epidemics worldwide without an available vaccine. The CHIKV are divided in two transmission cycles, the enzootic sylvatic cycle, and the urban one (Caglioti *et al.* 2013). One of the enzootic vectors is *Aedes furcifer* that may transmit the virus from monkeys to humans (Jupp and McIntosh 1990). Despite different species of *Aedes* being involved in the transmission of CHIKV, *Ae. aegypti* is one of the major vectors and maintains the virus in sylvatic areas in Africa, while in Asia CHIKV usually circulates in urban regions, which increases the spread between humans (Pastorino *et al.* 2004). Additionally, CHIKV has adapted to *Ae. albopictus* because of a specific mutation in the envelope protein of the virus (Tsetsarkin *et al.* 2007), increasing the viral fitness in this vector. During epidemic periods, humans act as the principal reservoir while monkeys, rodents and birds represent the main reservoir during non-epidemics episodes (Caglioti *et al.* 2013).

Dengue

Dengue Virus (DENV) causes more illness and deaths than any other arboviral infection in the world (Valentine *et al.* 2019). Although DENV is fully adapted to urban cycles and does not need a zoonotic reservoir, sylvatic cycles still exist. Dengue virus has been reported frequently in NHPs and in the mosquitoes that feed upon them (reviewed by Valentine *et al.* (2019)). Although the vectors of sylvatic dengue have been identified and spillover and spillback shown, little is known about their ecology and host preference. Valentine *et al.* (2019) describes a study in Malaysia in which the role of mosquitoes in sylvatic transmission was investigated. They isolated four DENV-types circulating between NHPs and mosquitoes. *Aedes niveus* was most likely the main vector in the sylvatic cycle as it preferentially fed on NHPs and in the forest canopy. Spillback of DENV from the urban cycle to NHPs has also been reported (Yuwono *et al.* 1984), but the mosquitoes that could facilitate such transmission have not yet been identified.

Malaria (Plasmodium)

Malaria is caused by *Plasmodium* parasites of which five species are known to infect humans. In NHPs a large diversity of *Plasmodium* parasites is documented of which most are host specific (Liu *et al.* 2010, Ngoubangoye *et al.* 2016, Sharp *et al.* 2020). *P. falciparum* is the most lethal human *Plasmodium* species and responsible for 228 million malaria cases and 405.000 deaths in 2018 (WHO 2019). In contrast to the other *Plasmodium* species, *P. falciparum* infection is commonly restricted to humans and has no zoonotic reservoir as it was not detected despite large sampling efforts across Africa (Liu *et al.* 2010, Prugnolle *et al.* 2010, Sharp *et al.* 2020). Nevertheless, *P. falciparum* has been found in NHPs living in sanctuaries or zoo's (Bakker *et al.* 2020, Ngoubangoye *et al.* 2016, Prugnolle *et al.* 2010). These findings, together with the results of experimental infections of chimpanzees with *P. falciparum* (Bray 1963, Taylor *et al.* 1985) suggest that cross-species transmission of *P. falciparum* to NHPs is possible but does not lead to a viable infection. Similarly, ape malaria parasites can also be transmitted from one ape species to another in a confined environment (Ngoubangoye *et al.* 2016). The concentration of different species of primates including humans may favour an exchange of pathogens and increase the risk of a spillover.

Mosquito species that could facilitate these exchanges are only studied occasionally and little is known about their host preference (Verhulst *et al.* 2012). Mosquito collections near wild and semi-wild ape populations in Gabon showed that *Anopheles moucheti*, *Anopheles vinckei* and *Anopheles marshalli* can probably support the sylvatic transmission of ape *Plasmodium* species (Makanga *et al.* 2016, Paupy *et al.* 2013). *Anopheles moucheti* is considered anthropophilic and is a vector of human malaria in forested areas (Fontenille and Simard 2004). If *An. moucheti* would also readily bite apes it could facilitate *Plasmodium* transfers between apes and humans, however, the highest prevalence of *Plasmodium* infection in the study of Makanga *et al.* (2016) was found in *An. vinckei*. This species was previously thought to be zoophilic (Gillies 1964) but human landing catches showed it would also bite humans if available (Makanga *et al.* 2016).

Host preference studies near a chimpanzee sanctuary in the Republic of Congo also revealed a mosquito population with a more generalised host preference, except for *Culex decens* which was more attracted towards chimpanzee odours than odours from other animals or humans (Bakker *et al.* 2020). In non-forested areas, mosquito host preference has a large effect on the transmission of *P. falciparum* and vector control methods against highly anthropophilic mosquitoes like *An. gambiae* are highly effective. In addition, laboratory experiments showed that anthropophilic mosquitoes prefer human above cow volatiles, but no preference was observed when human odour was tested against odours from different NHPs which could favour cross-species transmission (Verhulst *et al.* 2018). Temporal fluctuations of diversity and density of hosts in the forest may lead to a more opportunistic host seeking strategy (Makanga *et al.* 2016). Because small fluctuations in host preference may have a large effect on disease transmission these temporal fluctuations need to be studied in detail (Yakob *et al.* 2017). Future studies should reveal the significance of NHPs as a potential source for human infection with *Plasmodium* and the role of vector behaviour (Verhulst *et al.* 2012).

Plasmodium knowlesi can infect humans but has its main reservoir in long- and pig-tailed macaques (Moyes *et al.* 2014). Previously, *P. knowlesi* was not considered to be an important human parasite until a study in 2004 in Malaysian Borneo revealed a high prevalence of *P. knowlesi* in humans (Singh *et al.* 2004). Morphologically *P. knowlesi* is very similar to *P. malariae* and also the disease symptoms are very similar. Better PCR methods led to a better understanding of the distribution of this parasite and the underlying epidemiological factors. Transmission mainly occurs outdoor in the peri-domestic area (Manin *et al.* 2016) and indoor residual spraying and bednet distribution may therefore have less effect when compared to other *Plasmodium* species infecting humans. Deforestation causes the movement of macaques from forested areas to farms and semi-urban areas leading to an increased risk of transmission (Imai *et al.* 2014, Stark *et al.* 2019). Mosquitoes may have followed their hosts to this new habitat leading to an increasing risk for humans. Indeed, a case-controlled study in Malaysia that identified risk vectors for *P. knowlesi* infection revealed that the presence of monkeys nearby was a strong predictor of risk (Grigg *et al.* 2017). The current knowledge indicates that *P. knowlesi* exhibits unstable transmission in humans and therefore repeated invasion of the parasite into humans is expected (Yakob *et al.* 2017). The mosquito vectors that can facilitate this cross-species transmission have been identified and their behaviour studied occasionally. Biting hosts at ground level rather than high into the canopy seems to be important as this facilitates the biting of humans in the per-domestic area that are clearing vegetation or sleeping outside (Brant *et al.* 2016, Grigg *et al.* 2017). A biting preference for monkeys would facilitate the maintenance of a *Plasmodium* reservoir with an occasional spill-over to humans. Tsukamoto *et al.* (1978) showed that *P. knowlesi* carrying *Anopheles balabacensis* in the Philippines preferred monkey-baited traps above traps baited with water buffalo's or humans. However, this species is also known to readily bite humans (Brant *et al.* 2016) and therefore an efficient spill-over vector. In Malesia, Wharton *et al.* (1964) nicely showed that the presence of potential *Plasmodium* vectors depends largely on the habitat in which sampling takes place and at which height above ground. In addition, they found clear differences in biting preferences when bednet traps were baited with human or monkey odour. *Anopheles hackeri* was identified as the most important *Plasmodium* vector, carrying all the Malayan *Plasmodium* species and having a clear preference to bite monkeys with a man:monkey biting ratio of ratio of 100:819 (Wharton *et* *al.* 1964). Although mosquitoes with a preference for monkeys could facilitate the maintenance of a *Plasmodium* reservoir, opportunistic mosquitoes may be more important for cross-species transmission. *Anopheles latens* in Malaysia for example did not show a clear host preference and was attracted to traps baited with humans or long-tailed macaque monkeys in a 1:1.12 ratio (Vythilingam *et al.* 2006). The sporozoite rate was 1.18% indicating that *An. latens* could be an efficient bridge vector (Vythilingam *et al.* 2006).

After *P. falciparum, Plasmodium vivax* is the largest contributor to clinical malaria in the world and the most geographically widespread species (Battle *et al.* 2019). There is now growing evidence that *P. vivax* malaria is more widespread in Africa, but likely often undetected (Twohig *et al.* 2019). *P. vivax* appears to circulate freely among chimpanzees, bonobos and gorillas. The infection of a forester with *P. vivax* like primate parasites that probably originated from great apes (Prugnolle *et al.* 2013), shows that *P. vivax* lacks host specificity (Sharp *et al.* 2020). The frequency and mosquito vectors that could be involved in these cross-species transmissions remain to be investigated.

Plasmodium simium is genetically indistinguishable from *P. vivax* and has its reservoir in monkeys in South America (Lim *et al.* 2005). It is thought to ancestrally derive from a *P. vivax* host switch from humans to monkeys (Lim *et al.* 2005). Previously it was considered to be a monkey-specific malaria parasite, related to, but distinct from *P. vivax* (Brasil *et al.* 2017). However, a molecular epidemiological investigation after a malaria outbreak in Rio de Janeiro state revealed that all cases were shown to be infected with *P. simium* (Brasil *et al.* 2017).

Although malaria is normally not regarded to be a zoonotic disease, the examples above show that malaria parasites other than *P. falciparum* should receive more attention, especially when malaria elimination is the ultimate goal. Thorough screening of NHPs and mosquitoes is required to evaluate the extent of different *Plasmodium* species as a zoonotic threat to public health around the world.

Yellow fever

Although a vaccine against yellow fever virus (YFV) is available, outbreaks still occur, mainly in South America and Africa. Nearly all human outbreaks occur from spillover infections from NHPs and deaths of primates in South America are now a well-known warning sign of a YFV outbreak (Chippaux and Chippaux 2018, Valentine *et al.* 2019). Many different mosquito species have been identified as vectors of YFV including *Ae. aegypti* and *Ae. albopictus* (Barrett and Higgs 2007), but mosquitoes from the *Haemagogus* genus are probably most important as they maintain the sylvatic cycle of yellow virus in NHPs because of their high numbers and large distribution (Abreu *et al.* 2019). Species within this *Haemagogus* genus may bite at low and higher strata of the forest and both in the forest and at its edges, thereby facilitating the sylvatic cycle. Different *Aedes* mosquitoes, like *Ae. albopictus* have also been shown to be competent for transmitting YFV (de Oliveira *et al.* 2003), but they are little relevant as vectors because they do not bite in the forest, but mainly in cities and open fields (Abreu *et al.* 2019, Silva *et al.* 2020). Little is known about the role of mosquito host preference in the maintenance of the sylvatic cycle of YFV and the spillover to humans. Although the *Haemagogus* species are sometimes mentioned to be primatophilic and occasionally bite humans (Silva *et al.* 2020, Vasconcelos *et al.* 2001), the evidence for this is limited.

Zika

Zika virus (ZIKV) is a mosquito-borne *Flavivirus.* The first time that the virus was isolated was from monkeys in 1947 and from *Aedes africanus* mosquitoes in 1948, both in Uganda (Haddow *et al.* 1964), and later in humans in 1954 in Nigeria (Macnamara 1954). Predominantly, ZIKV has a sylvatic NHP cycle transmission, although humans can act as reservoirs, especially in urban areas (Musso and Gubler 2015). One of the most important risks of the infection is during pregnancy because ZIKV infection leads to an increased risk of microcephaly in the new-born (Mlakar *et al.* 2016), highlighting the importance of vertical transmission of ZIKV from mother to child. Although ZIKV was detected in *Aedes* mosquito species such as *Ae. africanus*, *Aedes luteocephalus*, and *Aedes taylori*, one of the most important vectors that acts as a bridge between the sylvatic areas and humans is *Ae. furcifer* (Diallo *et al.* 2014). In addition, competent vectors in urban areas are *Ae. aegypti* and *Ae. albopictus* (Musso and Gubler 2016).

Tools to determine host preference of mosquitoes in wildlife

Studies on host preference may be biased because of the limited availability of different hosts (Takken and Verhulst 2013). Mosquitoes may select a host that is readily accessible, although their inherent host preference is different (see Section *Host selection versus host preference*). To differentiate between host selection and host preference, the forage ratio can be calculated, which is the proportion of mosquitoes that fed on a given host, divided by the proportion of host species available in the mosquito's habitat (Hess *et al.* 1968, Jaleta *et al.* 2016). If the forage ratio is lower than one this indicates that the mosquito species avoids the host species while a forage ratio higher than one indicates preference.

Not only the availability of the host may have an influence on the host preference measured, but also the method that is used to define this preference. The proportion of human blood meals in mosquito abdomens is called the human blood index (HBI) and often used to measure the risk of disease transmission to humans in a certain area. Because it is difficult to catch blood fed mosquitoes with traps outdoors, in most studies mosquitoes were sampled indoors where they rest after their blood meal. This leads to an HBI that is often biased towards humans. When outdoor collections are included, the proportion of mosquitoes with a human blood is often much lower, although the mosquito species may be the same (Takken and Verhulst 2013). Catching blood fed mosquitoes outdoors can be done with resting boxes, (mechanical) aspiration and the use of barrier screens (Burkot *et al.* 2013, Qiu *et al.* 2007), although the mosquito catches with these methods are generally low. When host preference is determined by collecting blood fed mosquitoes, sampling should be performed indoors and outdoors and preferably with multiple collection methods. The forage ratio should be calculated and compared between mosquito species (Jaleta *et al.* 2016).

Choice tests to determine host preference may provide a good alternative to the collection of blood fed mosquitoes. Especially in the laboratory or semi-field, choice tests can easily be standardised. Nevertheless, working with life hosts in these settings is difficult. Host odour samples are therefore often used to determine host preference in laboratory, semi-field and field settings, but these samples may not include all host cues used by the mosquito to finds it blood meal. Outdoor catches with live hosts are probably the best way to determine host preference. Mosquitoes can be collected either directly from the host with aspirators or close to the hosts with traps or electric nets. Alternatively, hosts can be placed inside a double tent trap (Qiu *et al.* 2007). Nevertheless, outdoor catches with live hosts are not often used to determine host preference as the constrain of the animal requires ethical permission, is costly and often difficult to organise in wildlife settings.

Conclusions and further outlook

Spillover of pathogens from wildlife to humans and vice versa by mosquito vectors is still poorly understood. One of the reasons for this is that the current methods to determine host preference are difficult to apply in a wildlife setting. The ZIKA outbreak of 2015 showed that it is important to be able to monitor vector borne diseases in wildlife and its underlying mechanisms. ZIKA spread through an immunologically naïve population causing high morbidity. These new introductions of a pathogen in a naïve population are difficult to predict and depend on many different factors. Yakob *et al.* (2017) developed a mathematical model to predict the introduction of *P. knowlesi* into an immunologically naïve population. They showed that classic mosquito-borne disease model parameters like vector longevity were important, but also that host preference and especially behavioural plasticity can qualitatively impact the probability of an epidemic sparked by imported infection (Yakob *et al.* 2017). Once a pathogen has spread through a naïve population, wildlife may act as refugia for pathogens that may enable re-emergence once human epidemics have passed and there is fewer immunity in the population (Valentine *et al.* 2019).

Mosquitoes with an opportunistic feeding behaviour are probably the most important as spillover vector as they can transmit pathogens from one species to another. However, a more specific biting preference of the main vector may facilitate the maintenance of a reservoir with an occasional spill-over to humans, like seen for *P. knowlesi* transmitted by *An. balabacensis* (Tsukamoto *et al.* 1978) and WNV transmission by *Cx. pipiens* (Kilpatrick *et al.* 2006). A selective feeding preference for primates could then facilitate the spillover of vector borne pathogens between NHPs and humans. Although mosquito species have been identified that bite both NHPs and humans (Bakker *et al.* 2020, Makanga *et al.* 2016, Tsukamoto *et al.* 1978, Verhulst *et al.* 2018), little is known about their exact preferences, the underlying mechanisms, and their role in disease transmission between wildlife and humans.

Deforestation and an increased human population will lead to closer contact between wildlife, humans and the mosquitoes which will have to respond to this changing environment. Urbanisation for example is associated with an increased preference of *Ae. aegypti* for humans (Rose *et al.* 2020), which would increase the risk of vector borne disease transmission between humans but probably lower the risk of spillover by this mosquito species.

A deeper understanding of the processes and the biological interactions between the cues emitted by hosts and the response of mosquitoes may be used to improve mosquito traps and to develop novel methods and strategies in vector control. In addition, the role of mosquito host preference in the spillover of vector borne diseases is still poorly understood and should be improved. Although it can be expected that opportunistic mosquitoes play a more important role in the transmission between species, specialised mosquitoes can maintain a reservoir with an occasional spillover to other species. Monitoring at locations where spillover may occur, like forest edges, animal sanctuaries, bird refuges and zoo's, where there is a high concentration of animal species will help to detect spillover earlier. In addition, the identification of potential vectors and their behavioural characteristics with standardised methods will help to predict the risk of such spillover events.

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6. Tools for mosquito blood meal identification

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Abstract

The identification of the vertebrate blood meal sources of mosquitoes allows insight to better understand the dynamics of vector-borne pathogens. To do so, different approaches have been used, based on the use of the remains of blood present in the abdomen of recently engorged mosquito females. Among others, different authors have used serological techniques to the more recently developed approaches based on host DNA amplification or mass spectrometry. These methods have allowed researchers to identify the vertebrate hosts of mosquitoes accurately to the species level or, even, at the individual level, providing information on the relative importance of different mosquito species in the transmission of particular pathogens. These approaches have been especially relevant to reveal the contact rates between vectors, susceptible and competent hosts, and mosquito-borne pathogens, including zoonotic ones. Additionally, these methods have revealed important asymmetries in the attraction of mosquitoes towards different host species, allowing to identify key vertebrates for the amplification of some pathogens. This chapter reviews those tools most frequently used for the identification of the blood meals of mosquitoes in order to highlight the main advantages and limitations of these methodologies.

Keywords: barcoding, *cytochrome B*, COI gene, DNA, ELISA, Maldi-TOF

Introduction

The blood feeding behaviour of mosquitoes and their competence for the transmission of different pathogens make these insects one of the main vectors of causative agents of infectious diseases worldwide (Becker *et al.* 2010). Different species of mosquitoes of the genera *Aedes*, *Anopheles* and *Culex*, among others, are considered competent vectors of arbovirus including Dengue virus, Yellow fever virus, Zika virus or West Nile virus (Christophers 1960, Gutiérrez-López *et al.* 2019, Kilpatrick *et al.* 2008, Vasilakis *et al.* 2011). In addition, parasites including protozoan and filarial worms are important mosquito-borne pathogens affecting humans, livestock and wildlife (Christensen and Severson 1993, Valkiūnas 2004).

Mosquito females feed on blood to obtain the resources for egg development. Engorged female mosquitoes with a recent blood meal in their abdomen have been routinely used to identify the potential hosts of these insects in particular areas revealing that mosquitoes show different innate feeding preferences, with some species feeding mostly on mammals while others prefer to bite birds. In addition, different species of mosquitoes use ectotherms as the main blood meal sources, with records of species such as *Uranotaenia sapphirina* feeding on amphibians (Cupp *et al*. 2004) and *Culex hortensis* frequently feeding on reptiles (Martínez-de la Puente *et al.* 2015b).

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Table 1. Examples of studies using different molecular markers for the identification of blood meals of mosquitoes and other hematophagous arthropods.

Gene	Used in mosquito	Other insect used	Hosts identified by the studies	Reference
Mitochondrial DNA				
12S rDNA	not	ticks and Triatominae	mammals, birds, reptiles	Collini *et al.* 2015
COI	yes		vertebrates	Alcaide *et al.* 2009
Cytochrome b	yes		vertebrates	Fornadel *et al.* 2008
16S RDNA	not	biting midges and ticks	reptiles and amphibians	Collini *et al.* 2015
NADH dehydrogenase subunit (ND1)	yes		bats, reptiles and amphibians	Toma *et al.* 2014
Nuclear DNA				
Nuclear Ribosomal DNA (rDNA)	yes		annelids	Reeves *et al.* 2018
Prepronociceptin (PNOC)	not	biting midges	mammals	Hadj-Henni *et al.* 2015
Alu transposable elements	not	sand flies	humans and non-human primates	Siripattanapipong *et al.* 2018
Microsatellite	yes		mammals and birds	Martínez de la Puente *et al.* 2018, Yan *et al.* 2018

In addition, Miyake *et al.* (2019) found that *Aedes baisasi* includes fish as a common blood meal source in its diet. In spite of that, strong evidence support that many mosquito species show a relative opportunistic behaviour being able to feed on blood from different species or groups.

Authors have used different methods used for the identification of the vertebrate hosts of mosquitoes that include, among other, serological techniques, molecular approaches based on the amplification and sequencing of host DNA or mass spectrometry. Altogether, these methods have allowed researchers to accurately identify the vertebrate hosts of mosquitoes to the species level or, even, to the individual level (Alcaide *et al.* 2009, Beier *et al.* 1988, Gomes *et al.* 2001, Tandina *et al.* 2018, Yan *et al.* 2018). Thus, the identification of the vertebrate hosts of mosquitoes may be considered as an important step to evaluate the risk of transmission of mosquito-borne diseases (Coulibaly *et al.* 2016, Githeko *et al.* 1994, Ndenga *et al.* 2016). In particular, the identification of blood meal sources of mosquitoes allows researchers to infer the relative importance of different species of mosquitoes and/or vertebrate hosts in the transmission of particular pathogens by tracing the contact rates between vectors, susceptible and competent hosts, and mosquito-borne pathogens, including zoonotic ones (Ferraguti *et al.* 2020, Kilpatrick *et al.* 2006). These approaches may also allow researchers to identify the importance of particular individuals or groups within host populations (e.g. according to sex, infection status or odour) for the amplification of vector-borne pathogens (Lefèvre *et al.* 2010, Yan *et al.* 2018). These studies add valuable information to the traditional approaches based on the identification and counting of mosquitoes landing on the skin of animals (Balenghien *et al.* 2006, Ndenga *et al.* 2016) or to studies analysing mosquito attraction towards animals that are stalled in traps (Balenghien *et al.* 2006, Cozzarolo *et al.* 2019).

In this chapter, we review the more frequently used tools for the identification of blood meals of mosquitoes in order to highlight the main advantages and limitations of these methodologies.

Methods used to identify the blood meal sources of mosquitoes

Serological techniques

Techniques including the precipitin tests, such as haemoglobin crystallisation (Washino and Else 1972) and passive hemagglutination inhibition (PHI) (Kirsch and Murray 1969, Tempelis and Rodrick 1972, Weitz 1956), as well as enzyme-linked immunosorbent assays (ELISA) (Gentry *et al.* 1967, McKinney *et al.* 1972) have been widely used to identify the hosts of engorged mosquitoes. All of them basically consist in the identification of the host species of mosquitoes by exposing the blood meal to immunoglobulin G (IgG) conjugated against different potential host species (Mwangangi *et al.* 2003). Although this technique was firstly used to define the role of anophelines in the transmission of human malaria (Tempelis 1975), these studies have been extended to multiple species of mosquitoes of the genera *Aede*s, *Culex* or *Coquillettidia* (Gomes *et al.* 2001, Kurucz *et al.* 2006, Lorosa *et al.* 2010). In spite of that, studies using these methodologies have some limitations. For example, the accuracy of the host identification based on these methods may be limited by the availability of specific anti-sera against target species (Martínez-de la Puente *et al.* 2018). In addition, cross-reactions may occur in presence of blood from closely phylogenetic related species (Boakye *et al.* 1999, Hunter and Bayly 1991). This may limit the utility of this technique in studies performed under natural conditions where numerous vertebrate species are available to mosquito bites. For example, studies using serological techniques under natural conditions fail to identify the hosts of 13-20% of the analysed mosquitoes (Beier *et al.* 1988, Lorosa *et al.* 2010). Furthermore, although many studies are able to identify the avian origin of blood meals, the hosts species of mosquitoes are usually not determined (Lorosa *et al.* 2010). This fact together with the differential competence of different species of vertebrates for the development of pathogens, may limit the epidemiological conclusions obtained in these studies. An example of that could be the case of studies focus on WNV transmission, a pathogen which show a differential ability to develop in different species of birds (Linke *et al.* 2007, Llorente *et al.* 2013, Pérez-Ramírez *et al.* 2014). In example, host competence for WNV of passerines use to be higher than of galliformes and columbiformes (Pérez-Ramirez *et al.* 2014). *Culex quinquefasciatus* is an important vector for WNV, and different studies have shown preference to feed also on birds with a high capacity to amplify the virus (i.e. passeriforms) (Niebylski and Meek 1992, Zinser *et al.* 2004). However, it would be highly recommended to use methods allowing the identification of blood meals of mosquitoes beyond the ordinal level (i.e. species of passeriform), because such preferences may result in important differences in pathogen amplification (Kilpatrick *et al.* 2006).

Molecular approaches

The development of molecular techniques such as polymerase chain reaction (PCR) and sequencing has favoured the identification of vertebrate hosts of mosquitoes to species level (Kent 2009). These methods are based on the amplification of fragments of nuclear or mitochondrial genes of the vertebrate hosts using either species-specific primers or universal primers that are able to amplify the DNA of a wide range of vertebrates. Different molecular markers have been developed for this objective (Table 1; Borland and Kading 2021). However, the utility of these techniques is also limited by factors including the availability of enough well-preserved genetic material in the mosquito blood meal. The success of host identification decreases as the degradation of the blood meal increases as supported by studies on mosquito species including *Aedes aegypti*, *Anopheles atroparvus* or *Cx. quinquefasciatus* (Martínez-de la Puente *et al.* 2013, Oshaghi *et al.* 2006, Santos *et al.* 2019), although positive identifications are possible after 30-36 hours post ingestion (Oshaghi *et al.* 2006), or even, for up to 72 h after blood feeding (Ngo and Kramer 2003). Unfortunately, for the case of mosquitoes trapped under natural conditions, it is not possible to know the time spanned since blood meal ingestion to mosquito capture. In these cases, mosquitoes could be sorted according to their Sella score which consist of a scale from 1, corresponding to mosquitoes with a recent blood meal, to 7, corresponding to mosquitoes without visible blood and eggs fully developed in their abdomen (Detinova and Bertram 1962). This may be especially useful in wild-trapped mosquitoes, as the Sella scoring of mosquitoes' blood meals was negatively correlated with the success of host identification using DNA amplification and sequencing (Martínez-de la Puente *et al.* 2013, Santos *et al.* 2019).

Other potential limitation of molecular methods is the availability of reference libraries with the nuclear and/or mitochondrial gene sequences for the potential vertebrate hosts (Borland and Kading 2021). Mitochondrial DNA (mtDNA) remains a popular and advantageous sequencing target for arthropod blood meal identification (Kent 2009). A wide variety of animals are able to be identified by mtDNA markers including 12S and 16S Mitochondrial Ribosomal DNA (rDNA) (Collini *et al.* 2015), NADH Dehydrogenase Subunit I (ND1) (Toma *et al.* 2014), the cytochrome b (*cyt* b) and the cytochrome c oxidase subunit I (Cox1 or COI) genes, the last being the most frequently used (Alcaide *et al.* 2009, Kent 2009, Kent and Norris 2005). There are different public databases with information on the genetic sequences for many vertebrate species, including the Barcode of Life initiative, which use the COI gene as a barcode to genetically characterise the worldwide diversity of vertebrates (Hebert *et al.* 2003). Nowadays the Barcode Records on BOLD system (https://www.boldsystems.org/index.php) included 723,297 animal sequences corresponding to 45,744 species. Thus, identification of the vertebrate hosts of mosquitoes could be accurately reached by comparing the obtained sequence from the mosquito blood meals with those available in public databases. In those cases where there are not previous characterisations of the COI sequences of species, using these tools it is also possible to identify the hosts of mosquitoes at lower accurately taxonomic levels (e.g. Family). However, contrary to the case of the *cyt* B and COI genes, for other genes as the prepronociceptin gene (Pnoc) (Hadj-Henni *et al.* 2015) or Nuclear ribosomal DNA (rDNA) markers, the reference libraries available are limited (Reeves *et al.* 2018). In addition, the low variability in the Pnoc sequences of closely related vertebrate species, as in the case of horses and donkeys, may limit the accuracy of host identification (Hadj-Henni *et al.* 2015). This limitation should be considered together with the fact that mammal red blood cells are not nucleated, so the DNA available for molecular analyses based on the amplification of nuclear genes may be lower in these vertebrates compared to groups such as birds.

Mosquitoes´ blood meals from a single host are frequently reported, however mosquitoes are able to feed on different host individuals to complete their blood meals. Records of mixed blood meals exists for a number of species such as *Culex pipiens*, *Aedes albopictus*, or *Aedes japonicus* (Cebrián-Camisón *et al.* 2020) which include mosquitoes fed on different host species or on different individuals of the same species. In the case of molecular approaches, the analyses of these mixed blood meals may provide ambiguous DNA sequences with double peaks in the sequencing electropherograms. With the help of a reference database of potential vertebrate hosts and/

or the amplification of host DNA using specific-designed primers would be possible to identify bloodmeals from different hosts species (Alcaide *et al.* 2009, Kent 2009). This method could be also useful if intraspecific variability in the amplified region exists within species, as in the case of the different haplotypes of sheep identified in the blood meal of several hematophagous vectors in Spain (Calvo *et al.* 2012). However, when this is not the case, alternative methods could be used including the amplification and sequencing of highly variable DNA regions (i.e. microsatellites) at least, when information on the genetic characteristics of the potential hosts of mosquitoes is available (Yan *et al.* 2018).

Molecular fingerprinting based on the identification of host microsatellites have been used to accurately track the hosts of mosquitoes below the species level in studies of the transmission of pathogens affecting humans and other animals. For example, Scott *et al.* (2006) identified the differential susceptibility of humans within a population to the bites of human-malaria vectors. Similarly, these approaches have been also used in studies on the host selection of mosquitoes using different animal models including house sparrows (Yan *et al.* 2018) and pigs (Keven *et al.* 2019). Moreover, these approaches have been applied in studies on conservation biology, to track the individuals of endangered species such as the Iberian lynx (*Lynx pardinus*) fed by *An. atroparvus* in southern Spain (Martínez-de la Puente *et al.* 2015a). Because mosquitoes have a limited dispersal range after feeding on blood (for example, *Cx. quinquefasciatus* reported a maximum of 168 meters based on the study developed by Greenberg *et al.* 2012), this method may provide information on the home range of the target vertebrate individuals. Furthermore, the blood meals of mosquitoes can be also used to identify certain characteristics of their hosts. For example, Burkett-Cadena *et al.* (2014) found that *Culiseta melanura*, *Culex erraticus* and *Culex nigripalpus* female mosquitoes feed more frequently from male than female birds. Similarly, the molecular identification of the sex of vertebrate hosts provides information on host selection by mosquitoes which could be used to build epidemiological models of vector-borne diseases (Teltscher *et al.* 2021). This low-cost methodology allows researchers to study the role of sexual differences in the host utilisation by mosquitoes potentially explaining sex related differences in the incidence of vector borne pathogens.

One important challenge for studies on the identification of mosquito blood meals is the development of *omics* platforms to use in the field. For example, genomic technologies with field-adapted protocols and powerful miniaturised laboratories designed specifically for field deployment, such as MinION (Giordano *et al.* 2017, O'Guinn *et al.* 2004). This platform has demonstrated efficacy in *cyt b* sequencing from wildlife samples (Seah *et al.* 2020), thus being potentially useful to trace the hosts of mosquitoes.

Mass spectrometry associated techniques

Different mass spectrometry techniques have been recently used to determine the origin of arthropod blood meals, including mosquitoes. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) allows to identify host by specifically targeting proteins present in blood fed ticks and triatome (Keller *et al.* 2017, Önder *et al.* 2013). The obtained spectrum can be compared to theoretical spectra generated from known protein sequences and publicly available DNA and protein sequences in databases. Haemoglobin proteins are a well-studied and stable molecule showing an important potential for this technique, especially to analyse degraded insect´s blood meals. In addition, quantitative LC-MS/MS may allow researchers to quantify the amounts of haemoglobin and to detect potential mixed blood meals of mosquitoes (Gerber *et al.* 2003, Natarajan *et al.* 2015). However, this technique shows some important limitations including the availability of reference sequences of potential host species in published databases. In addition, haemoglobin proteins show high variability across vertebrates, showing some species polymorphic haemoglobin peptides which will reduce the resolution of host identification (Borland and Kading 2021).

The matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) has been recently used to assess the feeding patterns of mosquitoes. MALDI-TOF MS is based on a matrix of proteins that are ionised and, based on their mass-charge relationship, a specific spectrum profile of the analysed species is generated, capable of discerning even closely related species of primates or felines (Niare *et al.* 2016, Tandina *et al.* 2018). MALDI-TOF MS is a quick and easy method to analyse mosquito blood meals using currently available software (Niare *et al.* 2016). However, the use of this technique is limited by the absence of public profile databases. Nowadays, most laboratories build their own libraries considering a limited number of mosquito/host species (Niare *et al.* 2016, Tandina *et al.* 2018). This fact may compromise the use of these approaches for the study of mosquito blood meals of insects collected in the field (Niare *et al.* 2016). Furthermore, as previously discussed for the case of molecular approaches, the degradation of blood meals may affect the quality of the results obtained limiting the accuracy of host identification using MALDI-TOF MS (Niare *et al.* 2016). Vertebrate hosts of mosquitoes could be identified up to 24 h post ingestion, although factors including the storage of the samples could affect to the result obtained (Niare *et al.* 2016). Finally, it is important to highlight that MALDI-TOF have been also used for the identification of mixed blood meals of mosquitoes being able to detect the last and mixed blood meals of *Anopheles gambiae* and *Ae. albopictus* mosquitoes, although it was impossible to identify the first blood meal in successive blood meals (Tandina *et al.* 2020).

Additional information obtained from mosquito blood meals

In addition to reveal mosquito-host interactions, engorged mosquitoes can be also used to monitor the pathogens harboured by the hosts of these insect vectors. This approach known as xenosurveillance was defined as a new surveillance technique that utilises the mosquito blood meals to sample host pathogens (Grubaugh *et al.* 2015); that allows researchers to identify the contact rates of potential mosquito vectors and the pathogens which could transmit (Martínez-de la Puente *et al.* 2015b). These studies may be improved by the use of next-generation sequencing technology which is able to detect a high spectrum of pathogens could be used to detect all the potential pathogens harboured by mosquitoes that could be actively circulating in a particular area. On the other hand, mosquito blood meals could be used to obtain physiological information of the vertebrate hosts of mosquitoes. Blood meals of tse tse flies (Habicher *et al.* 2013) or kissing bugs (Voigt *et al.* 2006) have been used for the quantification of host´s hormones allowing, in example, the determination of pregnancy in female mammals. These approaches could be potentially used for the case of mosquitoes, although its application could be limited by the volume of the blood meal.

Concluding remarks

Mosquito blood meals provide a source of host material which could be used to identify the vertebrate hosts of mosquitoes up to the individual level. Different approaches have been developed during the last decades, with the molecular approaches likely being the most commonly used. However, the different techniques used to identify mosquito feeding have their advantages and disadvantages. Undoubtedly, the technique to be used in each case must be previously evaluated based on the availability of specialised laboratory equipment and resources, the quality and conservation of the samples and the accuracy of the required identifications. Different approaches could be combined to obtain the most valuable results. For example, the precipitation test such as ELISA can be combined with a subsequent PCR for the specific identification of bird species (Apperson *et al.* 2004). Likewise, given the limited number of species included in the databases of the spectrum generated by MALDI-TOF MS, molecular methods can be used to overcome this limitation, allowing greater specificity in the identification of the hosts of mosquitoes. Altogether, the information obtained from mosquito blood meals may provide a general overview of the importance of the different species of mosquitoes for the transmission of mosquito-borne pathogens of public and animal health relevance.

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Human role in the ecology of mosquito-borne diseases: global change effects

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7. Consequences of deforestation and habitat degradation on wildlife mosquito-borne diseases

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Abstract

Over the last several decades the rate of forest clearance has increased considerably. Millions of hectares of forests are lost every year for agricultural activities and timber extraction, which have significant consequences on atmospheric CO_2, climate change and ecological interactions. Many studies carried out in perturbed habitats or areas that have gone through significant habitat loss have reported changes in vector-borne diseases dynamics, with an altered abundance of both the invertebrate and vertebrate hosts that has led to changes in pathogen prevalence. Following deforestation, altered microclimates with changes in temperatures, sunlight, and moisture and the addition of artificial breeding habitats tend to favour common vectors of disease, such as species of the mosquito genera *Anopheles*, *Aedes* and *Culex*. Land conversion for agricultural use also provides increased accessibility to blood meals with domesticated animals breeding in close quarters of humans. In fact, approximately three fourths of human emerging infectious diseases are caused by zoonotic pathogens due to the increasing contact among humans, domestic animal populations and wildlife hosts. The malaria parasite *Plasmodium knowlesi* and the Mayaro virus are examples of mosquito-borne zoonoses that jumped recently from wild hosts to humans following their encroachment into tropical forests. However, at the moment, it is simply unknown how many pathogens are transmitted to wildlife by mosquitoes and while many studies have shed light on the dynamics of avian malaria or the West Nile Virus, many other parasites and the likely multitude of undiscovered viruses are still poorly studied. The reality is that with rapid environmental change, without in-depth research on mosquitoes that feed on wildlife and the pathogens they harbour, we will not be able to predict why diseases emerge and what factors exacerbate their spread.

Keywords: *Aedes*, *Culex*, human land-use, malaria, arboviruses, zoonoses

Introduction

In a very short time span, human-led changes, such as habitat destruction and climate change, have disturbed the natural stasis of most of the planet's ecosystems (Creutzig *et al.* 2019, He and Silliman 2019). In the last 40 years, natural vegetation, in tropical but also in the mid-latitudes, has been cleared at an arresting rate, millions of hectares per year, for agricultural activities such as support for livestock, monocultures and exotic tree plantations (Barlow *et al.* 2016, Tilman *et al.* 2001). Tropical forests, which account for one third of land-surface productivity and evapotranspiration (Malhi 2012), are estimated to host over half of all global terrestrial biodiversity (Pimm and Raven 2000) and are key to ensure many of the planet's vital functions. Since the second half of the past century, these ecosystems have been constantly shrinking. The latest data show that the tropics lost 11.9 million hectares of tree cover in 2019, with nearly a third of that loss, 3.8 million hectares, occurring within humid tropical primary forests (Global Forest Watch

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2019). This habitat loss is the result of increasing impacts of multiple human activities, such as logging and other resource extraction (Malhi *et al.* 2014), livestock pastures (Almeida *et al.* 2016a) and conversion to agricultural use (Laurance *et al.* 2014). Among several crops, the cultivation of the oil palm *Elaeis guineensis* has been expanding at an annual rate of 9% worldwide (Vijay *et al.* 2016) and this rapid expansion has resulted in a significant loss of biodiversity and associated ecosystem services (Almeida *et al.* 2016b, Ganser *et al.* 2017, Savilaakso *et al.* 2014). In temperate areas, natural forests have also been intensively harvested and dramatically reduced, mainly due to overexploitation and conversion to farmland and tree plantations (Hannah *et al.* 1995, Teixido *et al.* 2010). As an example, the spread of the exotic tree species, *Eucalyptus globulus* has transformed the landscape of the Atlantic coast of Northern Iberia and is impoverishing regional avifauna and the communities of other taxonomic groups (Calviño-Cancela *et al.* 2012, Proença *et al.* 2010). In addition to solely habitat loss, habitat fragmentation is a consequential process that subdivides continuous habitats into smaller pieces and results in population isolation and a disruption in landscape connectivity (Fahrig 2003). Therefore, not only forests are under major threats, but also other biomes are in peril; e.g. the Cerrado in South America, which is considered one of the most important savanna biomes in the world, with a mixture of open grasslands, shrub lands, open woodland and closed canopy woodland (Bridgewater *et al.* 2004). This ecosystem is undergoing pervasive degradation because of agriculture expansion, with vast areas recently modified for soy cultivation (Lima *et al.* 2019), which has put wildlife populations at risk (Borges *et al.* 2019).

All these human activities and habitat degradation are expected to disrupt ecological interactions, notably relationships between hosts, vectors and pathogens, and to influence the dynamics of transmission of infectious diseases (Faust *et al.* 2018, Sehgal 2010, Walsh *et al.* 1993; Figure 1). There have been many examples of direct effects of deforestation on human pathogens (e.g. malaria: Burkett-Cadena and Vittor 2018, Yanoviak *et al.* 2006, Yasuoka and Levins 2007). Increased levels of sunlight, associated with open spaces, have been correlated with increased mosquito densities and feeding rates of mosquitoes on humans and increased parasite transmission (Vittor *et al.* 2006, 2009). However, some studies have also reported no obvious link between deforestation and the prevalence of vector-borne diseases (see examples in review Tucker Lima *et al.* 2017). These conflicting findings are likely attributable to the complexity of vector-borne disease systems, and depend on the study region and associated socio-ecological factors (MacDonald and Mordecai 2019). As with infectious diseases in humans, numerous studies focusing on avian malaria have found conflicting results on the impacts of deforestation and fragmentation (Chasar *et al.* 2009, Loiseau *et al.* 2010, Rivero de Aguilar *et al.* 2018, Tchoumbou *et al.* 2020). Interestingly, although the diversity of mosquitoes that feed on birds can be higher in degraded environments, some studies also found a lower abundance in urban habitats (Abella-Medrano *et al.* 2015, 2018) or fragmented forests (Tchoumbou *et al.* 2020) compared to well-preserved forests.

Environmental changes are therefore modifying transmission dynamics of pathogens specific to humans or to wildlife, but another consequence of deforestation and encroachment into wildlife habitat, and associated activities such as hunting, wildlife trade, live animal and bushmeat markets (Symes *et al.* 2018), is the risk of the emergence of zoonoses (Jones *et al.* 2013). A zoonosis is any bacterial, viral or parasitic disease that is naturally transmissible from animals to humans. Approximately three fourths of human emerging infectious diseases are caused by zoonotic pathogens (Taylor *et al.* 2001) and because humans and domestic animal populations come into increasing contact with wildlife hosts harbouring potentially zoonotic pathogens, a large increase of zoonotic pathogens emergence has been reported in the last decades (Swei *et al.* 2020). Zoonoses can be transmitted directly from animals to humans, such as Ebola (Buceta and Johnson 2017), Nipah virus (Gurley *et al.* 2017), or the novel SARS-CoV-2 (Andersen *et al.* 2020),

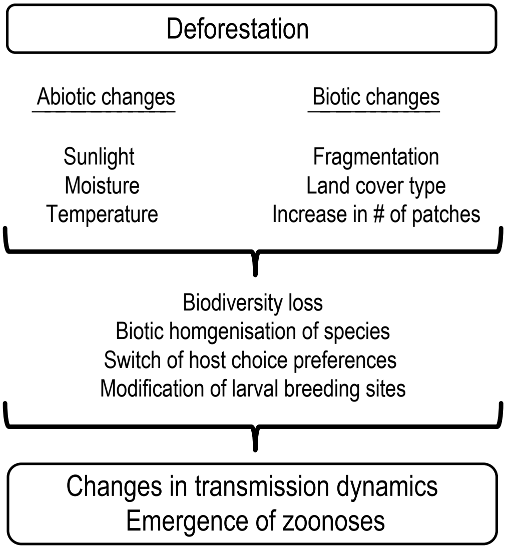

Figure 1. Diagram of deforestation consequences on mosquito-borne diseases.

or transmitted via an arthropod-vector, such as Zika virus (Saiz *et al.* 2016) and West Nile virus (Tolsá *et al.* 2018) among others. Here, we will attempt to evaluate and give an overview of the impacts of deforestation, habitat fragmentation and degradation (1) on mosquito communities (i.e. diversity, abundance, and assemblage), (2) the consequences on mosquito-borne disease specific to animals, and (3) the consequences on mosquito-borne zoonotic diseases. Finally, we address potential challenges working with mosquito-borne diseases and recommend future lines of research.

How are mosquito communities affected by environmental degradation?

Given the unprecedented global effects on the environment and biodiversity worldwide, distributions of vector-borne diseases are similarly changing radically. For mosquitoes, important components for their survival are temperature, the availability of water and plant resources, plus the necessity of blood meals for females. With global climate change, deforestation, urbanisation, and other changes in land use by humans, mosquito populations and the pathogens they transmit are undergoing shifts that may have long-term effects on wildlife and humans. As with biodiversity worldwide, we can expect a homogenisation of niches (Clavel *et al.* 2011), resulting in a loss of habitat specialist mosquitoes and the global proliferation of generalists and invasive ones, such as *Aedes albopictus* (Niebylski *et al.* 1994), which is a major human disease vector.

Globally, with more than 3,500 species, mosquitoes are among the most successful insect groups on the planet, with their richness increasing along a gradient from the higher latitudes toward the equator (Foley *et al.* 2007). About 50% of the species are endemic, but only 3% or about hundred species actually feed on humans (Foster and Walker 2019). With global climate change, the general expectation is that mosquitoes will move into the higher latitudes, following rising temperatures. With deforestation, urbanisation, and loss of wetlands and other habitats, we expect an overall reduction in mosquito species diversity, and a general trend towards the spread of invasive mosquitoes and the loss of endemics (Brady and Hay 2020, Manguin and Boëte 2011). This will be most apparent on islands, and is punctuated by the classic example of the invasion of the Hawaiian Islands by mosquitoes in the 19th century (Keyghobadi *et al.* 2006). One important understudied aspect of mosquito entomology is how the loss of many endemic mosquito species will shape ecosystem services, such as plant pollination (Lahondère *et al.* 2020), the filtering of water by consuming microorganisms and detritus (Merritt *et al.* 1992) or the survival of other threatened species that could be dependent on them as a food resource (Blaustein and Chase 2007, Poulin *et al.* 2010). The loss of endemic species could in turn lead to an increase in the invasive mosquitoes that may replace them (Johnson and Sukhdea 2013). Since there is a general disdain for mosquitoes by humans, as they are certainly not charismatic megafauna, mosquito conservation biology has not been a very active area of research. However, it is likely that the loss of many endemic mosquito species will lead to ecosystem deficits, and perhaps the loss of other dependent organisms (Fang 2010).

With the demise of such a substantial portion of the planet's native habitats due to human encroachment, habitat generalist mosquitoes are increasingly spreading across the planet. With the mosquitoes, pathogens will follow. It has been shown that several invasive mosquitoes have recently moved into habitats that were once dominated by endemics. The yellow fever mosquito, *Aedes aegypti*, is one of the most dangerous mosquitoes to humans due to its capacity to transmit several debilitating viruses, such as the Dengue, Chikungunya as well as Zika and Yellow fever, among others. It is believed that this mosquito species, up until as recently as 500 years ago was entirely a forest-dwelling mosquito that fed on non-human mammals (Powell *et al.* 2018). As humans moved into its native habitat, it quickly adapted to using human water containers for its larval stages, and began to preferentially feed on humans (Brady and Hay 2020). It began to spread worldwide with the movement of ocean-going ships. In particular, it most likely arrived in the New World with the slave trade (Powell *et al.* 2018) and is now spreading worldwide (Akiner *et al.* 2016, Liu-Helmersson *et al.* 2019). With its capacity to transmit dangerous flaviviruses and alphaviruses, it already has killed untold numbers of humans (Brady and Hay 2020) and has also affected wildlife (e.g. West Nile, Rift Valley, Usutu viruses; Bird *et al.* 2009, McLean *et al.* 2002, Roesch *et al.* 2019). Similarly, *Anopheles stephensi*, which transmits human malaria in Asia has expanded its range recently, and was discovered in Sri Lanka for the first time in 2017, most likely arriving with humans from India (Sinnathamby *et al.* 2019). These are just two of the numerous documented cases of mosquito vectors expanding their ranges with human populations.

The majority of habitats on the planet are experiencing rapid environmental changes, where some of the major contributing processes can be classified by deforestation, urbanisation, agricultural development and at the larger scale, global climate change (Franklinos *et al.* 2019). It is clear that biodiversity is rapidly lost with forest clearance (Newbold *et al.* 2015), and can result in the emergence of mosquito-transmitted diseases. Following deforestation, changes in microclimates (temperatures, sunlight, and moisture) and the addition of artificial breeding habitats tend to favour vectors of disease, such as species of *Anopheles*, *Aedes* and *Culex* (Burkett-Cadena and Vittor 2018, Mayi *et al.* 2019). Similarly, land conversion for agricultural use, in addition to providing more suitable habitats for human disease-carrying mosquitoes, may also provide increased accessibility to blood meals with domesticated animals breeding in close quarters (Service 1991). Most humans now live in urban environments, and this results in altered climates through the creation of urban heat islands, which again favour the urban vectors of the genus *Aedes* (Murdock *et al.* 2017), but may hinder the development of *Anopheles* species, which transmit malaria (Qi *et al.* 2012).

The effects of global climate change on mosquito populations are complex, and there is no general consensus regarding how changes in climatic conditions will impact the risk of mosquito-borne diseases (Franklinos *et al.* 2019). Some climate scenarios will result in better habitats for mosquitoes, and others will hinder their survival. The effects of increasing temperatures on insects are expected to be non-linear, where some mosquito populations will benefit and others may suffer from reduced fitness, such that certain areas of the planet may actually see reduced numbers of mosquito-borne disease cases (Mordecai *et al.* 2017). It is most straightforward to surmise that climate change will have unpredictable effects on mosquitoes, since they are so closely tied to the often capricious behaviours of humans (Bartlow *et al.* 2019). We can be certain however that environmental change will alter the patterns of transmission, in humans, domestic animals and wildlife.

How does environmental degradation alter infectious disease dynamics in wildlife?

As stated earlier, deforestation and habitat fragmentation affect the diversity and abundance of mosquitoes. Mosquitoes are known vectors for many pathogens, and while they are well known to transmit human diseases such as dengue, Zika, malaria, lymphatic filiariasis and others, it must be remembered that in studying the ecology of disease, research on human diseases can be confounded by human socioeconomic conditions and health interventions (MacDonald and Mordecai 2019, Tucker Lima *et al.* 2017). For example, human malaria was at one time prevalent in many parts of the world, including North America, but was eradicated with the advent of pesticides, and improvements in anti-malarial drugs (Andrews 1948, Carter and Mendis 2002). Thus, studying the ecology of mosquito-borne diseases of wildlife can give a better indication of the true effects of rapid environmental changes and deforestation on disease transmission.

Over the last decades, avian malaria has become a trenchant model system to research how habitat changes can affect the prevalence and diversity of vector-borne pathogens in wildlife (Ferraguti *et al.* 2020, Sehgal 2015). We have learned that pathogen dynamics are highly complex. First, the prevalence of avian malaria (of the genus *Plasmodium*), and other related haemosporidian parasites, varies tremendously with habitat and bird communities. In terms of deforestation, studies have reported contrasting results for *Plasmodium* parasites, with either an increase in prevalence in disturbed habitats (Chasar *et al.* 2009, Hernández-Lara *et al.* 2017, Reis *et al.* 2020, Tchoumbou *et al.* 2020), a higher prevalence in forests (Bonneaud *et al.* 2009, Loiseau *et al.* 2010) or no impact of the land use type (Rivero de Aguilar *et al.* 2018, Sebaio *et al.* 2012). Recently further work has shown that the prevalence of avian malaria is significantly and positively correlated with mosquito abundance in a fragmented African rainforest (Tchoumbou *et al.* 2019; Figure 2), although it is apparent that this type of association can be readily affected by the seasonality and again the species, or lineage, of the parasite (Fecchio *et al.* 2017, Lalubin *et al.* 2013). Similarly, the diversity of the parasites varies with changes of habitat. As is typical with biodiversity in general, deforestation and loss of rich diverse habitats result in a decreased diversity of avian malaria parasites, and this can be expected due to the loss of locally endemic bird species and mosquito vectors (Sehgal 2015). Moreover, we hypothesise that with deforestation and habitat loss, there will be a loss of specialist parasites, and generalists will prevail. This could eventually lead to the dominance of generalists such as the parasite *Plasmodium relictum,* which caused the large-scale deaths of birds when it invaded the Hawaiian Islands (LaPointe *et al.* 2012). In effect, we can expect more generalist malaria parasites to emerge with associated rapid environmental change, which may contribute to the loss of many endemic or immunological naïve bird species.

Figure 2. Picture of a CDC light trap installed in a palm oil plantation to collect vectors and identify their associated diseases in highly disturbed habitat after deforestation. © Rafael Gutiérrez-López.

It is likely that deforestation and land use changes will affect the prevalence of malaria in many other animals besides birds. It is now known that malignant human malaria (*Plasmodium falciparum*) originated in gorillas (Liu *et al.* 2010), and many primates are frequently infected with various species of primate malaria (Prugnolle *et al.* 2010). With the persistent human encroachment into areas frequented by primates, it is likely that human *Plasmodium* parasites may more readily be transmitted between humans and non-human primates (Duval *et al.* 2010, Liu *et al.* 2010, Loy *et al.* 2017). Primates are threatened more now than ever in their long history because of the extensive habitat loss, increased bushmeat hunting and illegal trade (Estrada *et al.* 2017). With altered mosquito communities, deforestation will also make them more susceptible to human mosquito-borne pathogens. As we now know, many human pathogens have a sylvatic cycle, and mosquito populations with access to both primate and human communities will undoubtedly contribute to more disease in both wildlife and humans (Valentine *et al.* 2019). For example, the Yellow Fever virus is greatly impacting primate populations in South America (Holzmann *et al.* 2010). New World monkeys suffer habitat destruction and hunting pressure, which fragments populations. They are in addition highly vulnerable to this virus, which is almost always fatal, and therefore, outbreak events of yellow fever are another major threat to their survival (Agostini *et al.* 2015). Interestingly the humans and monkeys are probably only temporary amplifiers of yellow fever, since studies showed that mosquitoes could be the primary reservoirs, as they are infected throughout their lives, with maternal transmission from adults to eggs (Tomori 2004).

Today, it is simply unknown how many pathogens are transmitted to wildlife by mosquitoes. Certainly, many studies have shed light on the dynamics of avian malaria, the West Nile Virus (Blitvich 2008, Morin and Comrie 2013), and to a lesser extent avian pox (Atkinson and LaPointe 2009), but many other parasitic pathogens, such as nematodes and trypanosomes and the likely multitude of undiscovered viruses are relatively unstudied. Thus, the reality is that with rapid environmental change, without in-depth research on mosquitoes that feed on wildlife and the pathogens they harbour, we will not be able to predict why diseases emerge and what factors exacerbate their spread.

What are the consequences of deforestation on zoonotic diseases dynamics?

Vector-borne zoonoses represent today a problem of increasing importance since they are almost impossible to eliminate. Indeed, there is no way to control all the natural reservoir hosts (wild birds and mammals) that are part of sylvatic cycle of numerous viruses and eukaryote parasites that infect humans. However, the risks of an infectious disease jumping from wildlife to humans can be reduced by investigating and knowing: (1) the diversity of wildlife pathogens in a region, which is considerably more important in the tropics than in temperate regions, (2) the effects of environmental changes on wild populations and on the distribution of vectors and (3) the frequency of contacts between humans and wildlife (Wolfe *et al.* 2005).

In 1993, Walsh *et al.* wrote one of the first reviews about the effects of deforestation on vector-borne diseases. The authors described the discovery of a tick-borne zoonosis in the tropics: Kyasanur Forest Disease (KFD). At first, in South India, languor monkeys, *Semnopithecus entellus*, and macaque monkeys, *Macaca radiata*, were found dead in a forested area, infested with ticks. Not long after, researchers identified a new virus, and then, on a quite small geographical scale, every year, new cases were reported as well as the deaths of people in the nearby villages. Monkeys, small mammals such as rats, mice and shrews, as well as birds were found to act as amplifier hosts, and to carry infected ticks in villages (Hoogstraal 1966). The KFD is one of the examples of diseases that came into contact with human population following ecological changes, namely deforestation and wildlife habitat perturbation. There are many additional examples in the literature of the effects of deforestation, habitat fragmentation and global changes on a wide array of vectors and their associated pathogens (Colwell *et al.* 2011). Here, we choose to focus on zoonotic mosquito-borne diseases caused by two different pathogens: *Plasmodium knowlesi* and the Mayaro virus, found in Southeast Asia and the Americas respectively. These two pathogens are still restricted to their original regions, as opposed to others such as the West Nile or Dengue viruses that are now cosmopolitan, and therefore they require heightened vigilance to avoid uncontrolled spread.

P. knowlesi malaria is a mosquito-borne zoonosis present in Southeast Asia, which infects primarily long-tailed, *Macaca fascicularis*, and pig-tailed macaques, *Macaca nemestrina* (Eyles *et al.* 1962) and was identified as an emergent public health threat only recently in 2004 (Cox-Singh and Singh 2008, Singh *et al.* 2004). This simian *Plasmodium* is transmitted by various forest-dwelling mosquitoes such as *Anopheles balabacensis*, which is considered the primary vector in Sabah (Wong *et al.* 2015) but also *Anopheles cracens* (= *An. balabacensis balabacensis*) in Peninsular Malaysia (Vythilingam *et al.* 2008), *Anopheles hackeri* (Wharton and Eyles 1961) and *Anopheles latens* in Sarawak, Malaysia (Vythilingam *et al.* 2006). Several studies have shown that changes in forest cover have impacted vector abundance and assemblages, as well as disease prevalence in humans (Brock *et al.* 2019, Hawkes *et al.* 2019, Wong *et al.* 2015) but very few have investigated the effects of deforestation and habitat fragmentation on macaque populations and their movement and distribution. It was suggested that deforestation may cause macaques to crowd into the remaining forest patches, spend more time on the ground (Riley 2008, Singh *et al.* 2001) and seek crops and food around human settlements (Hambali *et al.* 2012). These behavioural changes, subsequent to habitat perturbation, are resulting in an increased contact rate between macaques, humans and *Anopheles* mosquitoes, and therefore an increase in spillover events from macaques to humans is expected. In fact, experimental work in the laboratory has shown that *P. knowlesi* could be transmitted via mosquito bites from monkey to monkey, from monkey to humans, from humans to humans and from humans back to monkeys (Chin *et al.* 1968). In addition, *Anopheles* mosquitoes that feed on both macaques and humans have been found to harbour several other simian parasites (e.g. *Plasmodium cynomolgi*, *Plasmodium coatneyi* and *Plasmodium inui*; Wong *et al.* 2015), which might be of concern to humans in the near future (Raja *et al.* 2020). Taking into account the ecological requirements of each host through an integrative approach, at both the small and large-scale, is necessary to define areas at risk for this zoonotic mosquito-borne disease (Brock *et al.* 2016, Davidson *et al.* 2019).

The Mayaro virus is an Alphavirus of the family Togaviridae, such as Chikungunya and Sindbis viruses (Lavergne *et al.* 2006). This virus circulates in Central and South America in humans and causes acute febrile illness (Anderson *et al.* 1957), which is difficult to differentiate clinically from Chikungunya or Dengue (Tesh *et al.* 1999). It was first isolated from sick forest workers in Trinidad and Tobago in 1954, then in Colombia, Brazil, Suriname, Guyana, Peru, Bolivia (see references in Tesh *et al.* 1999), French Guiana (Talarmin *et al.* 1998) and Venezuela (Torres *et al.* 2004). Interestingly, a very diverse group of wild vertebrates have been found with anti-Mayaro virus antibodies: lizards (Taylor 1967), birds (Calisher *et al.* 1974), several primate species (Hoch *et al.* 1981, Seymour *et al.* 1983, Talarmin *et al.* 1998), but also marsupials, sloths, rodents and carnivores (De Thoisy *et al.* 2003). Studies found that, in general, animals that are diurnal and live in the canopy tend to have a higher prevalence of the virus than others (De Thoisy *et al.* 2003, Talarmin *et al.* 1998), which corresponds to the niche of the mosquito genus *Haemagogus*; the main vectors of the Mayaro virus. These diurnal mosquitoes are tree-dwellers, very common in rainforests (Hoch *et al.* 1981), and are rather opportunistic feeders, which can explain the large variety of vertebrate hosts infected, including humans (Alencar *et al.* 2005).

People living within or in close proximity to forests appear to be the most affected by the disease (Black *et al.* 1970, Talarmin *et al.* 1998) and an increasing number of human cases in the Amazon basin seem to be due to ecosystem disturbances. Since the 1950's, regular outbreaks have occurred in Brazil but not only in the northern region where the disease is endemic: this raises concerns about the spread and transmission of the virus to areas outside of the Amazon basin (Esposito and Fonseca 2017). Several monkey species are quite common near villages and cities, and could act as reservoirs to an urban transmission cycle, in addition to the birds that may also have a role in the natural transmission of the virus (Hoch *et al.* 1981). Today, of primary concern are the peri-urban mosquito species that have been found naturally infected with Mayaro virus (*Mansonia venezuelensis*, Aitken *et al.* 1960; *Culex vomerifer*, Galindo and Srohongse 1967) and others that have been found as competent vectors in the laboratory such as *Ae. aegypti* and *Ae. albopictus* (Smith and Francy 1991, Long *et al.* 2011). The extensive deforestation in South America and urbanisation, coupled with the colonisation of this expanding habitat by the anthropophilic mosquitoes, *Ae. aegypti* and *Ae. albopictus* will probably lead to movement of the Mayaro virus outside the sylvatic cycle, as did Chikungunya or Dengue (Mackay and Arden 2016, Weaver and Reisen 2010). These two viruses lost the requirement for wildlife amplification and have now an efficient human-mosquito-human transmission. When and where this could happen with Mayaro virus remains difficult to predict.

Challenges and future research

Researchers studying mosquito-borne diseases in wildlife face several challenges. First, in tropical regions, there is an overwhelming diversity of mosquito species with complexes of species often extremely difficult to identify. The description of new species requires advanced entomological expertise and today there are very few researchers that have practice identifying mosquitoes of little medical or veterinary importance. One avenue to enhance the knowledge of the diversity of mosquitoes in remote regions, especially in tropical rainforests, is through the use of new methodological tools, such as metabarcoding of eDNA samples (Ji *et al.* 2013, Ruppert *et al.* 2019). In fact, collecting water where larvae grow is relatively straightforward and this technique has shown promising results (Boerlijst *et al.* 2019, Krol *et al.* 2019). However, the issue remains that database references are incomplete and that in depth taxonomy work is still necessary to match DNA sequences to species identifications.

In addition, researchers often lack information about the feeding habits of mosquitoes, which is essential to understand the transmission dynamics of diseases. To evaluate how disturbances will influence the host choice of vectors and eventually the switch of pathogens from one host species to the other, the analysis of blood meals is critical (Stephenson *et al.* 2019). However, sampling blood-fed mosquitoes is a challenge in itself, even for medical entomologists. Blood-fed females, depending on the species, rest on the vegetation, near the ground or in the canopy and therefore canopy-dwelling mosquito species are nearly impossible to catch. Still, improved rope-climbing methods (Picart *et al.* 2014), cranes (Nakamura *et al.* 2017) or original airborne devices and canopy rafts have been created (Charron *et al.* 2020, Gottsberger 2017) to allow scientists to access forest canopies and unravel their amazing biodiversity; richer than in all the other forest strata.

Encouraging collaborations among researchers with complementary expertise is essential. Since many pathogens infect wildlife and humans, entomologists, ecologists, wildlife conservationists, parasitologists and sociologists should work together to better understand how deforestation and habitat disturbances affect the diversity of mosquito species and the dynamics of their associated diseases. In the early years of the 21st century, 'One Health' was proposed as a concept to foster interdisciplinary collaborations. This approach promotes integrative studies to tackle complex, interconnected human – animal – ecosystem disease problems (Gibbs 2014). Multi and transdisciplinarity between life sciences, social sciences and the humanities is required, but researchers, human and animal health organisations are sometimes facing institutional and logistical barriers to achieve such an approach (Waltner-Toews 2017). Although predictive maps of infectious disease emergence are regularly produced (Allen *et al.* 2017, Messina *et al.* 2019, Wilkinson *et al.* 2018), who knows how many zoonoses the world will face in the next decades. In the meantime, mosquitoes keep swarming and spreading while sadly humans keep destroying forests.

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8. Implication of human landscape transformation on mosquito populations

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Abstract

Anthropogenic landscape transformation has an important effect on vector-borne pathogen transmission, especially under the perspective of an increase in their distribution and incidence in recent decades. Drivers of the increase of vector-borne diseases are attributed to global change including, but not limited to, habitat alteration and simplification of habitat structures such as increase of urbanised areas, alteration of trophic interactions, the introduction of invasive exotic species, or climate change. However, the effects of urbanisation on mosquito communities are still only poorly known and there is relatively little understanding of how human-induced rapid global change is impacting the dynamics of vectors and vector-borne diseases. Urbanisation is especially significant in the case of mosquitoes, one of the most nuisance animals associated with human activities with a high medical relevancy. For most of this vector species, the abundance and species richness are considered lower in urban areas than rural or natural ones, mainly due to anthropogenic activities that produce substantial ecological disturbances that can affect animal communities at both levels of vertebrate and invertebrate organisms. However, container-breeding species, like introduced *Aedes* exotic mosquitoes, or group of species as the *Culex pipiens* complex, seem to benefit from specific urban features where there is an abundance of artificial oviposition sites. Moreover, some forest mosquito species, such as *Aedes japonicus*, may also spread into urban areas due to the expansion of peri-urban habitat into suburban growth areas that increasingly invade more transitional and disturbed rural areas, as for example in allotment gardens or cemeteries. To sum up, studies on mosquito populations from urban areas and surrounding anthropized environments, suggest an overall community-level response to urbanisation. Furthermore, research considering both the land cover heterogeneity of the urban environment and the assemblage of insect species are essential to develop adequate strategies against emerging vector-borne diseases. This chapter focuses on summarising recent works that investigate the effects of urbanisation on insect vector community ecology.

Keywords: *Aedes* exotic mosquitoes, *Culex*, exotic species, mosquito community composition, natural-rural-urban gradient, urban ecology, vector-borne diseases, vector community ecology

The urban expansion: effects on vector community

Nowadays vector-borne diseases are a worldwide public health concern since both their distribution and incidence are increasing in recent decades, with significant wide-ranging economic implications (Kilpatrick and Randolph 2012, World Health Organization 2017). Despite more than 700,000 people die from vector-borne diseases each year, unfortunately, the magnitude of this

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problem has only recently become apparent when the United States has doubled the reported number of vector-borne diseases between 2004 and 2016 (Rosenberg *et al.* 2018). Mosquitoes (Diptera: Culicidae) are one of the most nuisance animals associated with human activities with a highly medically relevance (Tolle 2009, World Health Organisation 2014). With over 3,500 species worldwide, these insects are by far the most common vectors of numerous pathogens including metazoan, protozoan and numerous viruses (Tolle 2009), causing millions of deaths per year which add up to global economic and health care burdens (World Health Organisation 2014).

In the last century, human rural population has gone from 6.7 inhabitants to each urban dweller to less than one (Satterthwaite *et al.* 2010). Human-induced landscape alteration has rapidly increased during the last decades, and cities around the world are becoming a common feature of landscapes (Alberti 2008, Forman 2014). Two-thirds of the human population will be living in cities by the year 2050, imposing environmental demands at different spatial and temporal scales (Grimm *et al.* 2008, McDonnell and Pickett 1990, Montgomery 2008). Due to major landscape modifications related to the settlement of novel urban structures and to cover human basic needs (e.g. food, energy, water; McDonnell and Pickett 1990), urbanisation has been identified as a major threat to biodiversity (Alberti 2008, McKinney 2002, Shochat *et al.* 2010), directly affecting more than 3,000 species listed as threatened or near-threatened according to the IUCN Red List (Maxwell *et al.* 2016). Indeed, anthropogenic activities produce substantial ecological disturbances that affect animal communities at both levels of vertebrate and invertebrate organisms (McKinney 2008), and often lead to an increase in the abundance of a few species and a general loss of biodiversity (McKinney 2002). In the case of pathogen vectors, this is important due to the increased dominance of a few key species, and the ecological conditions (e.g. anthropized environments) could favour their interspecific contact rates (i.e. increased biting frequency) and pathogen transmission rates (Woolhouse *et al.* 2001). This can lead also to a process of homogenisation through the introduction of non-native species (McKinney 2008), an issue of particular relevance if we focus on the invasive species vector of pathogens of clinical relevance. However, there is still relatively little understanding of how human-induced global change, and especially urbanisation, is affecting the dynamics of vector-borne diseases.

Urban ecosystem characteristics

Factors including the simplification of habitat structures (Shochat *et al.* 2010) and the alteration of trophic interactions (Faeth *et al.* 2005) may govern anthropogenic-mediated loss of biodiversity in urban areas. Changes in resource availability, vegetation coverage, the characteristics of water bodies, and both temperature and rainfall patterns, may all have a severe impact on mosquito populations (Becker *et al.* 2020, Li *et al.* 2014), thus directly and/or indirectly affecting their community ecology (McKinney 2008) and the pathogen transmission risk (reviewed in LaDeau *et al.* 2015).

The fast development of cities has provoked the expansion of the urban habitat to the suburban growth areas that subsequently leaded to an increase of urbanisation (Abdullah and Hezri 2008, Holway and Suarez 2006, Riley *et al.* 2005), where the forested areas of the surrounding agriculture lands were in close contact with the industrial and residential properties (Abdullah and Hezri 2008). This expansion could have favoured the arrival of new rural insect vectors in peri-urban areas, such as the emblematic case of *Aedes japonicus* (Bartlett-Healy *et al.* 2012, Kampen and Werner 2014). Thus, urban environments such as parks, roadside green, built surfaces or refuse heaps, could become hotspots supporting the invasions of invasive species in cities (Gaertner *et al.* 2017, Kaufman and Fonseca 2014) (Figure 1).

Transitional areas between different land-use types, such as along a natural-rural-urban gradient, generally have a special significance for the occurrence of adult mosquitoes due to their high habitat structural diversity (Reiskind *et al.* 2017). Spatial gradients in transition zones have an influence on biotic and abiotic conditions such as microclimate (urban areas can be cooler at the edge of the city, compared to the interior) or biotic factors (more artificial breeding sites could be found inside the cities for *Culex* than at the edge). Here, the importance of these areas for vector species lies in the very diverse microclimatic and habitat conditions they can provide, which creates emerging interfaces for interaction between humans and other species (Vanwambeke *et al.* 2019). Depending on the container type (e.g. tree hole at the base of a trunk), water temperatures can be much lower than the air temperature, resulting in microhabitats offering shelter from extreme temperatures (Scheffers *et al.* 2014). On the other hand, independent of the dimension of climate change scenarios common urban species such as *Culex pipiens* s.l. will generally benefit from warm water temperatures in natural or artificial containers. For instance, these microclimate refugia can be widespread in urban areas and can be exploited by different mosquito species, or species biotypes such as *Cx. pipiens molestus*. The microclimates can be caused by urban heat-island effects or by different urban infrastructures and can provide shelter to this species on underground locations of the metropolitan areas. This contrasts with the case of other species including *Ae. japonicus* that appears to spread and establish where settlements meet areas with small patches of forests.

Impacts of habitat disturbances on insect vectors, hosts and pathogens

Changes in land use, such as the introduction of human habitations and agriculture into previously undeveloped areas that are enzootic for several zoonosis, could promote interactions among vectors and hosts, increasing the likelihood of sylvatic-pathogen re-emergence (Patz *et al.* 2004). For instance, habitat alteration, such as that resulting from deforestation, has been linked to the increased prevalence of several mosquito-borne diseases, such as malaria (Olson *et al.* 2010, Vittor *et al.* 2009), although it could also limit the circulation of enzootic pathogens by reducing the suitable environment for some mosquito vectors.

In many tropical regions, mosquitoes represent a problematic issue, especially related to urban landscapes where their abundance increases (Gubler 2011). This is also affected by the rapidly changing, interconnected world where we are living. Transport connections have improved the possibilities for movement of many insect vectors, particularly mosquitoes and ticks, creating novel scenarios whereby established exotic vectors out of their native areas have facilitated transmission of previously tropical and subtropical pathogens (Medlock *et al.* 2012). This may explain the occurrence of different outbreaks of imported diseases such as dengue and chikungunya in Europe during the last years. In particular, outbreaks of chikungunya have been reported in Italy (Angelini *et al.* 2007), several dengue cases have occurred in countries including Italy, France, and Spain (Dalla Pozza and Majori 1992, La Ruche *et al.* 2010, Redondo-Bravo *et al.* 2019), and the recent emergence of West Nile fever in the south of Spain, reminding us of the risk of imported pathogens (Rodríguez-Alarcón *et al.* 2021). In these cases, invasive species such as *Aedes albopictus* are suspected to play a central role in these transmission events.

The current situation has been aggravated by the fast and continuous expansion of urban areas, connecting cities with altered environments where vector ecology patterns and disease transmission dynamics have been affected by land-use intensification and habitat alteration (Patz *et al.* 2004, Sala *et al.* 2000). The likelihood of a pathogen to jump from one host species to another is a function of both the ecological and spatial proximity and the phylogenetic relationship between the donor and recipient species. Hence, the alterations in land use, expanding travel networks or increases in human population size and density, could favour cross-species transmission of viruses and other zoonotic pathogens to humans. For instance, the cross-species transmission of (non-vector related) immunodeficiency viruses from non-human primates to humans has been associated with the rise of the logging industry in Central Africa (Wolfe *et al.* 2005). The increasing urbanisation of some areas of Asia and Africa where sylvatic dengue virus circulates, could lead to larger populations of *Aedes aegypti*, potentially increasing the risk of exposure of people to sylvatic strains and favour the occurrence of human-mosquito-human transmission events (for a review see Vasilakis *et al.* 2011). Also, the recent introduction and expansion of *Ae. albopictus* in Africa (Lambrechts *et al.* 2010, Paupy *et al.* 2009) may increase the risk for dengue emergence from sylvatic foci. Indeed, this vector has already mediated important epidemics of chikungunya virus in Africa in a human-mosquito-human cycle. Nowadays, coinciding with the human and economic impact of Covid-19, Latin America is additionally facing the most severe dengue epidemic in history, with a total number of cases exceeding by 30% the previous record of 2015 (Stanaway *et al.* 2016, World Health Organization 2021).

Effects of anthropogenic changes on mosquito population

Urbanisation is one of the anthropogenic changes that greatly impacts the epidemiology of mosquito-borne diseases (Johnson and Munshi-South 2017, Rosenberg *et al.* 2018), and leads to serious outbreaks even in previously non-endemic areas (Gould *et al.* 2017, Poletti *et al.* 2011). Recent studies have shown that landscape changes contribute to an alteration at a local scale in the distribution and abundance of mosquito species (e.g. Abella-Medrano *et al.* 2015, Ferraguti *et al.* 2016), and hence as a by-product, may affect the spread of pathogens, including zoonotic ones (Norris 2004, Patz *et al.* 2000). Understanding how environmental variables influence mosquito distribution is therefore an urgent need in disease ecology (Sehgal 2015).

Urban habitat provides alternative artificial breeding sites for mosquitoes, such as water deposits, available water-filled containers in gardens and subterranean water systems. This is especially the case during the dry season when surface water is otherwise scarce. In fact, several species of *Culex* (Byrne and Nichols 1999), *Aedes* (Kay *et al.* 2000) or *Anopheles* (Overgaard *et al.* 2003) are favoured by the effects of water accumulation related to urban settings. Urbanisation ecosystem, in addition to providing suitable habitats for mosquito breeding, also offers a large availability of human hosts and a considerably reduced number of predators (Jia *et al.* 2017, Johnson and Munshi-South 2017).

Urban infrastructures such as airports and harbours play an important role in the introduction and dispersal of novel mosquito species (Ibáñez-Justicia *et al.* 2020a). Globalisation has increased this phenomenon, bringing mosquito species to other adjacent regions, or from continent to continent. Due to the large number of travellers and goods transported, and the frequency of the delivery, blood seeking mosquitoes can enter in ships, containers or planes and be unnoticed transported to other areas. Recent examples related to aircraft movements are *Ae. aegypti, Ae. albopictus, Anopheles subpictus,* and *Mansonia* sp. in the Netherlands, and *Anopheles pharoensis* in Belgium (Ibañez-Justicia *et al.* 2017, 2020b). Container carriers are considered as an important point of entry of exotic mosquitoes due to their international movement of goods. Indeed, the international maritime trade nowadays accounts for the 90% of the global exchange of goods in the world (ICS 2020). Considering the presence of rainwater drainages with stagnant water, these ships can provide potential breeding and shelter sites for mosquito species, such as *Ae. albopictus* that was introduced in Mediterranean countries (Di Luca *et al.* 2017). Additionally, travels by car may also contribute, albeit to a lesser extent, to the dispersal of mosquito species within countries (Eritja *et al.* 2017).

Community composition and abundance in urban areas

The list of mosquito species that are likely to be favoured by anthropogenic environmental change comprises *Anopheles darlingi* (Jones, 2003), *Anopheles gambiae* (Frankie and Ehler, 1978; Nelson, 1978), *Ae. aegypti* (Frankie and Ehler 1978; Nelson 1978), *Anopheles albitarsis* and *Culex nigripalpus* (Forattini and Massad 1998). The abundance and species richness of mosquitoes are generally lower in urban areas than rural or natural ones, as supported by studies conducted in Europe (Ferraguti *et al.* 2016, Ibañez-Justicia *et al.* 2015) and Australia (Johnston *et al.* 2014) (Figure 1). However, the effects of urbanisation on mosquitoes may vary between mosquito species and localities. For example, while mosquitoes of the *Mansonia* genus predominated in rural habitats, in urban sites *Culex* mosquitoes are usually more common (Johnson *et al.* 2008). Also, higher species richness has been found near urban environments (i.e. peri-urban forests) or within urban greenspaces, though abundance was higher at the better-preserved natural areas in Mexico (Abella-Medrano *et al.* 2015, 2018). By contrast, a negative association between mosquito abundance and richness and urbanisation was found in a Mediterranean area from Spain (Ferraguti *et al.* 2016) affecting the transmission of mosquito-borne pathogens (Ferraguti *et al.* 2018). One important variable affecting both mosquito abundance and richness was human population density, with drastic changes in mosquito density at areas with over 50 inhabitants/250 m^2, approximately (Ferraguti *et al.* 2016). Nevertheless, the negative effect of human population abundance on mosquito density and their effects on the transmission of vector-borne pathogens have received further support which may be driven by the implementation of insect control programs in the cities (Padmanabha *et al.* 2012). In a recent study, highly urbanisation negatively affected the distribution and abundance of the common house mosquito *Cx. pipiens*, while moderately and less urbanised areas were more suitable for this species (Gangoso *et al.* 2020). This pattern might be due to the semi-natural water sources in suburban areas which provide propitious conditions for mosquito breeding and maintenance, together with access to abundant vertebrate hosts, including humans. However, urban ecosystems are not homogeneous across the world, especially in developing regions, which can explain large variation in distribution patterns found in literature (Abella-Medrano *et al.* 2015, 2018, MacGregor-Fors and Escobar-Ibáñez 2017).

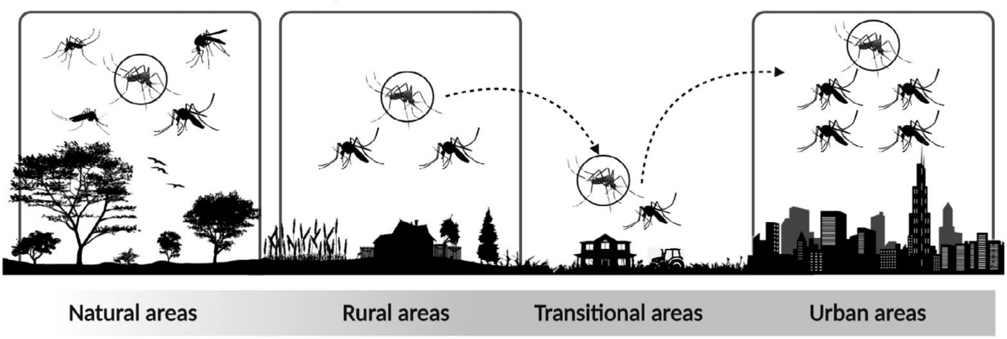

Figure 1. The expansion of anthropic environment into natural areas favours the arrival of new rural mosquito species, such as Aedes japonicus *(in the circle), along a natural-rural-urban gradient. In the natural habitat, both the abundance and richness of mosquitoes is higher than in urban environments.*

Likewise, high abundances of several mosquito species were found further from the city centre and closer to saltmarshes (i.e. rural areas) in South Australia (Johnston *et al.* 2014). Salt marshes provide a suitable environment for those salt-loving mosquito species (halophilic) such as *Culex modestus* and *Aedes* (*Ochlerotatus*) *caspius*, and strongly affect their abundance (Leisnham and Sandoval-Mohapatra 2011; Johnston *et al.* 2014). Particularly worthwhile is the example of species from southern Spain where the relationship between mosquito abundance and distance to marshlands was not linear, showing a marked threshold at distances of about 2.5 km for *Cx. modestus* and 10 km for *Ae.* (*Oc.*) *caspius* (Ferraguti *et al.* 2016). These differences fit well with the estimated mosquito flying distances, which range were found between 0.16 and 1.98 km for *Culex* (Ciota *et al.* 2012) and up to 12 km for *Ae.* (*Oc.*) *caspius* (Bogojević *et al*. 2011). Similarly, research have demonstrated that mosquito presence is positively associated with higher vegetation coverage (Vezzani *et al.* 2005, Zhou *et al.* 2007). As an example, in the city Tucson (USA), higher mosquito activity was recorded specially in residential neighbourhoods, areas typically characterised by higher vegetation coverage (Landau and Van Leeuwen 2012).

Urban community gardens and the spread of **Aedes** *invasive mosquitoes*

In recent years, an urban feature with leisure function for citizens has been pointed as a hotspot mosquito breeding: allotment gardens. Allotments are defined as 'a collective of garden plots that lie adjacent to each other, effectively subdividing a larger piece of land' (Bellows 2004). Community gardens are cultivated either communally or individually by a group of people from the direct neighbourhood or the wider city, in which urbanites are involved in other ways than gardening. The main purpose of the allotment gardens is growing your own plants for food. During the last decade, urban agriculture and the placement of community gardens in cities has become very popular in many European countries (Veen 2015). Examples of the current importance of community gardens can be found in England, Italy, or Spain. Indeed, in the British capital, community gardens occupy about 831 ha of public land, 13.4% of which is located inside the urban area and the remainder in the outskirts of London (Crouch 1997). In Bolonia, in the last 3 decades, the urban agriculture it has extended around 29 ha, including urban gardens (16 ha) and other garden typologies (13 ha, e.g. private gardens, gardens in monasteries, schools, illegal squatted gardens) (Bisetti *et al.* 2014). Finally, in the city of Valencia, the effect of the financial crisis also acted as a catalyst for urban greening latent demands, causing an overall expansion of the community gardens (Palau-Salvador *et al.* 2019).

The transformation of wasteland into community gardens can be beneficial against mosquito populations and mosquito-borne diseases, as in many cases, vacant lots overgrown with weeds and bushes can hide empty containers that can shrubs mosquitoes. However, to take care of plants during dry periods, or at allotments without irrigation systems, gardeners often collect water in containers such as rain barrels or buckets. Also, there are numerous water-butts and watering cans with stagnant water on allotments, which can provide an ideal habitat for mosquitoes breeding in the containers. Due to the presence of water-filled containers and vegetation, abandoned and overgrown allotments can become a perfect breeding ground for mosquitoes, which could eventually increase the risk of mosquito-borne disease outbreaks.

Container-breeding species seem to benefit from specific urban features where artificial oviposition sites are abundant (Honnen and Monaghan 2017), such as in allotment gardens or cemeteries (Vezzani 2007). Container-breeding mosquitoes are a well-defined group characterised by the properties of their habitats, and containers comprise a distinct group with unique ecological properties (Washburn 1995). As proposed in the review by Vezzani (2007), compared to pools, ponds, swamps, and rice fields, containers could be described as follows: (1) they are significantly smaller and will therefore support fewer species with smaller population sizes and exhibit higher extinction rates, (2) they are habitats with almost zero internal productivity, based mainly on the decomposition of leaf litter and other detritus, and (3) their larval mosquito populations are regulated by food limitation or competitive interactions rather than predation.

In Europe, several indigenous mosquito taxa have been found associated with water-filled containers present in home gardens, including *Aedes*, *Anopheles*, *Culex* and *Culiseta*. In addition, allotment gardens have provided the first evidence of the presence of a mosquito species (*Culiseta longiareolata*) in Germany in 2011 (Becker and Hoffman 2011). In the Netherlands, *Culex* sp. larvae were the most abundant in containers in the allotments of the city of Lelystad (83.93%), followed by *Ae. japonicus* larvae (14.63%), and *Culiseta* sp. (1.31%). Larvae of *Anopheles* sp. and *Aedes/ Ochlerotatus* were also found to a lesser extent (Ibañez-Justicia *et al.* 2018). The common house mosquito *Cx. pipiens* is a common mosquito species in allotments (Becker *et al.* 2020), easily found in large numbers and involved in West Nile virus (WNV) transmission in Europe. However, few investigations have studied its abundance and proliferation in allotment gardens.

Allotment gardens are also an excellent breeding site for introduced *Aedes* exotic mosquito species with invasive potential. In the Netherlands, following the discovery of a breeding population of the Asian bush mosquito (*Ae. japonicus*) (Ibañez-Justicia *et al.* 2014), a control campaign was planned in 2015 in heavily infested allotment gardens. In the first measurement before the control campaign, 29.95% of the containers present in the allotments contained larvae of *Ae. japonicus* (Ibañez-Justicia *et al.* 2018). *Ae. japonicus* was found in a wide variety of different containers, such as buckets, small bins, watering cans, wheelie bins, cement mixing containers, tarpaulins, and used tires. However, compared to the other potential breeding sites, *Ae. japonicus* larvae were most frequently found in rain barrels. Due to the efficiency of detection of *Ae. japonicus* larvae in allotment gardens, the availability of these urban features in the country and the high number of accessible breeding sites, monitoring allotments in adjacent municipalities was recommended to rapidly detect the spread of the species from Lelystad to the rest of the country.

The Asian tiger mosquito (*Ae. albopictus*) has also recently been associated with infestations in allotment gardens in Germany (Pluskota *et al.* 2016; Becker *et al.* 2017). Following a tip-off from an alerted gardener in an allotment garden area in Freiburg in 2015, a surveillance programme was started focusing on the garden and adjacent areas as well as most cemeteries as possible infestation areas (Becker *et al.* 2017). The surveillance programme confirmed a high infestation of the allotment garden, with the container index (CI) being almost 30% in August 2015. In Heidelberg, as part of a larval surveillance, the first larvae hatching in the field were detected in April in a rain barrel in an allotment garden (Pluskota *et al.* 2016).

One of the strongest assets in the fight against container-breeding mosquitoes is community involvement (Becker 1992), therefore mosquito control in allotment gardens needs the participation of the gardeners. For example, proper cleaning of containers seems to be effective in combating *Ae. albopictus* in Germany (Becker *et al.* 2017). Indeed, in Freiburg, a public awareness campaign was conducted to encourage gardeners to remove unnecessary breeding sites, teaching them to store buckets under roofs or to turn them upside down so that rainwater did not accumulate in them. In addition, modifications were also made to different structures suitable as breeding places with well-fitting nets or lids, such as covering the containers together with taping small holes in the cover. All these practices were well accepted by citizens responsible for the urban gardens (Becker *et al.* 2017).

Classic vs newcomer mosquito species in urban areas

Urbanisation is a continuous process, transforming and creating new habitats over time. Urban ecosystems can effectively act as a filter that regulates vector species composition due to the differential ecological requirements that have been reported within mosquito species (Becker *et al.* 2020). Indeed, mosquito species with different ecological requirements, life histories and morphological traits respond differently to habitat fragmentation related to urban areas. Most species that thrive in urban environments are considered generalists in term of habitat and diet. This is the case for the invasive species *Ae. albopictus*, which is able to colonise both natural breeding grounds as rock pools, tree holes, etc., and man-made sites, such as rainwater manholes vases, barrels, tires, etc. This mosquito is capable of biting different animal hosts in the absence of humans. Urban areas are usually associated with greater selective pressure on specialised organisms than on generalists, although this varies from taxon to taxon (Adams *et al.* 2020, Faeth *et al.* 2011, McIntyre 2000). Furthermore, anthropogenic landscapes can be expected to favour some mosquito species over others (Câmara *et al.* 2020, Rose *et al.* 2020). Densely populated settlements may contribute to the abundance and development of synanthropic organisms due to the presence of urban green spaces that provide mosquitoes with ample shelter and a variety of food sources (Barredo and DeGennaro 2020, Medeiros-Sousa *et al.* 2015). Mosquito species strongly associated with natural habitats can show a strong decline in urban environments, except if these are well connected to natural green areas. Areas with high fragmentation and where urban and rural habitats are adjacent and well connected to natural habitats may allow typical salt marshes or forest mosquitoes to be found in parks in cosmopolitan cities, such as Amsterdam (the Netherlands) (Ibañez-Justicia *et al.* 2015). Finally, those species associated with edge habitats thrive and adapt to intermediate environmental conditions (Frankie and Ehler 1978, McKinney 2008) such as *Ae. japonicus* in Europe. In Germany, the transition zone between forest and settlements was demonstrated to be the preferred oviposition habitat of *Ae. japonicus* and the presence of large areas of arable land represented distribution barriers for this species (Früh *et al.* 2020).

The Culex pipiens *complex in urban habitats*

The *Cx. pipiens* complex includes six distinct taxa (or subspecies) that are well adapted to urban habitats worldwide. These taxa include the cosmopolitan northern (*Cx. pipiens pipiens*) and southern (*Cx. pipiens quinquefasciatus*) house mosquitoes, the 'London Underground Mosquito' (*Cx. pipiens molestus*), a taxon endemic to northeast Asia (*Cx. pipiens pallens*) and two taxa endemic to Australia (*Cx. pipiens australicus* and *Cx. pipiens globocoxitus*; Aardema *et al.* 2020). In a recent modelling study conducted in Spain (Gangoso *et al.* 2020), the presence of this species complex has been mainly linked to human-related activities and infrastructure variables. A strong positive association was found with the degree of imperviousness, which can be considered as a proxy for the amount of urbanisation that discriminated between built-up and unbuilt-up areas (Gangoso *et al.* 2020).

Species of this complex are the main vectors of WNV and St. Louis encephalitis, among other pathogens (Becker *et al.* 2020). Here, we will focus on *Cx. pipiens molestus* (hereafter referred as *molestus*). Across most of its range, especially in temperate regions, *molestus* is highly adapted to urban environments and shows extensive ecological divergence from its sister taxon, *pipiens*, with which it is able to hybridise (Martínez-de la Puente *et al.* 2016, Vinogradova 2000). In addition to its differential habitat requirements, the *molestus* form, or biotype, differs essentially from its surface-dwelling relatives by its (1) willingness to mate in confined spaces (stenogamy), (2) ability to lay eggs without a previous blood meal (autogeny), (3) absence of diapause, and (4) variation in host feeding preferences (Aardema *et al.* 2020). All these characteristics can be related to their adaptation to a subterranean life: they mate in enclosed areas and lose their tendency to hibernate in winter, as there is no evident season underground. In addition, underground mosquitoes are not as abundant as above ground ones and do not form large swarms for mating and reproduction. Finally, while the above ground *pipiens* relatives preferentially bite birds, the *molestus* form has an interesting taste for human blood, which explains its name for its tendency to be 'molested'. Indeed, under an epidemiological perspective, it has been proposed that mosquitoes of the *molestus* form feed mainly on mammals (Fritz *et al.* 2015, Osório *et al.* 2014), while hybrids may have an intermediate feeding behaviour, potentially playing a role in the transmission of pathogens between humans and birds (Fritz *et al.* 2015), such as the WNV. Interestingly, a study conducted in Spain found no significant differences in the feeding patterns of *Cx. pipiens* forms, with birds dominating the diet of *molestus*, *pipiens* and their hybrids. The authors thus reported a similar contact rate between mosquito forms and the avian parasites *Plasmodium* and *Haemoproteus* (Martínez-de la Puente *et al.* 2016).

Today, there is no consensus on the evolutionary origins of *molestus*. It is unclear whether *molestus* mosquitoes are an urban form of *pipiens* that arose when *pipiens* forms adapted to urban and underground environments, or whether they have distinct evolutionary origins (Aardema *et al.* 2020). The *molestus* form from northern European countries clearly prefers to live in cities and subterranean habitats, such as the London underground railway tunnels (Byrne and Nichols 1999). In fact, the tunnels of the London underground system were sealed from the surface, which probably caused the divergent evolution of mosquito populations trapped underground. In Mediterranean countries, mosquitoes of both forms (*pipiens* and *molestus*) are sympatric, and they have often been found to be able to hybridise with each other (Amraoui *et al.* 2012, Di Luca *et al.* 2016, Gomes *et al.* 2009, 2013, Krida *et al.* 2015). Numerous studies on natural hybridisation have been conducted in Europe (Danabalan *et al.* 2012, Di Luca *et al.* 2016, Dörge *et al.* 2020, Gomes *et al.* 2009, 2013, Martínez-de la Puente *et al.* 2016, Osório *et al.* 2014, Reusken *et al.* 2010, Rudolf *et al.* 2013, Vogels *et al.* 2015, Zittra *et al.* 2016) and around the world (Beji *et al.* 2017, Weitzel *et al.* 2009; and for a review, see Fonseca *et al.* 2004).

The invasive **Aedes** *species: the case of the Asian tiger mosquito*

The Asian tiger mosquito has been listed by the Invasive Species Specialist Group as one of the 100 most invasive exotic species in the world (Lowe *et al.* 2000), representing a major threat to public health (Medlock *et al.* 2012). Therefore, considering the great invasive potential of this vector species, it is urgent to understand future transmission patterns of vector-borne pathogens with a wide range of economic implications (Bonizzoni *et al.* 2013). In its native range in Southeast Asia, *Ae. albopictus* is considered a rural species due to its preference for natural breeding sites and its occurrence at forest edges (Higa 2011). However, during the last decades this species has been introduced dramatically to all continents except Antarctica (Bonizzoni *et al.* 2013, Sherpa *et al.* 2019), due to human transport (Eritja *et al.* 2017, Medley *et al.* 2015, Reiter 1998). Maritime transport of tyres and other goods is suspected to have played an important role in supporting the spread of this mosquito across continents and some environmental factors probably aided establishment and further spread (Caminade *et al.* 2012). A study by the European Centre for Disease Prevention and Control (2013) showed that the mosquito's presence is mainly driven by an increase of rainfall and diurnal surface temperature. In fact, under the perspective of warming climate, models predict a continued northward expansion (Caminade *et al.* 2012, Ibañez-Justicia *et al.* 2020a), placing the European continent at particular risk (Fischer *et al.* 2011).

The success of these invasive mosquitoes, which seem to prefer anthropized areas to natural landscapes in their range, lies in their ability to use small accumulations of water, such as those formed in flowerpots or sewers. Indeed, like all other *Aedes* mosquito species, *Ae. albopictus* needs stagnant water for larvae development and has been able to spread through the trade of used ornamental plants and tyres or by passive transport in cars, being favoured so efficiently by urbanisation processes (Eritja *et al.* 2017, Roche *et al.* 2015). Their presence can be also favoured by urban ecosystems, including the aforementioned allotments, urban zoos (Martínez-de la Puente *et al.* 2020) or linked structures such as sustainable drainage systems used to manage stormwater (Valdelfener *et al.* 2019). In its natural native region, where the host community included a high density of birds, the percentage of avian-derived blood meals of the Asian tiger mosquitoes can reach up to 70.0% (Hess *et al.* 1968). Interestingly, in urban habitats, *Ae. albopictus* females avoid avian hosts (Martínez-de la Puente *et al.* 2015) and prefer to feed on mammals (e.g. domestic animals, such as dogs and cats), but mainly show an anthropophilic feeding preference (Faraji *et al.* 2014). Based on their trophic preferences and given the low percentage of avian blood in their meals, the importance of this mosquito species for the transmission of pathogens circulating between birds and mammals (e.g. WNV, avian malaria parasites) can be considered low (Martínez-de la Puente *et al.* 2015). However, this species may represent a key potential vector for the transmission of human pathogens such as chikungunya virus (Paupy *et al.* 2010), dengue virus (La Ruche *et al.* 2010, Paupy *et al.* 2010), zika virus (Grard *et al.* 2014, Gutiérrez-López *et al.* 2019) and *Dirofilaria* (Cancrini *et al.* 2003, Gratz 2004). In a scenario of global change, this mosquito species involved in the transmission of several pathogens definitely deserves further studies.

Concluding remarks and future research directions

Studies that take into account both the heterogeneity of the urban environment and the assemblage of urban insect species are essential for developing appropriate strategies against mosquitoes and vector-borne diseases. To date, studies on mosquito populations in cities and surrounding anthropized environments suggest an overall community-level response to urbanisation. Abundance and richness of native species tend to increase from city centres to peri-urban and rural surroundings. However, many biotic and abiotic factors associated with urbanisation can create contrasting responses depending on the mosquito community abundance and species composition.

It is important to remember that the establishment of an exotic vector species can create new epidemiological scenarios in the invaded range, with important ecological and health consequences for humans, wildlife, and domestic animals. However, disease incidence is not only affected by vector distribution, but also by other factors such as human activities, including public health policies, or geographical overlap between pathogen-reservoir vectors and humans (Gething *et al.* 2010, Martínez-de la Puente *et al.* 2018), all of which contribute to the uncertainties inherent in predictions about future changes in the distribution of vector-borne diseases.

Future research should focus on (1) identifying associations between mosquito community composition and abundance related to urban characteristics (e.g. parks, greenspaces, heat island, commercial areas, etc.), (2) characterising new hotspots for mosquito populations in European cities, and (3) studying the consequences of changes in urban plan designs on vector-borne diseases transmission (e.g. the investment in more green spaces, reduction of rain drains, improvement of urban sewers). This will provide insight into how anthropogenic activities modify the ecological dynamics of host-vector-parasite interactions.

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9. A meta-analytic approach to investigate mosquitoes' (Diptera: Culicidae) blood feeding preferences from non-urban to urban environments

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Abstract

Anthropogenic impacts modify host-parasite interactions and increase the probability of pathogen spillover across the human-domestic-wildlife interface. Important human emergent diseases are vector-borne and have a zoonotic origin. The mosquitoes (Diptera: Culicidae) are a family of blood sucking insects and their feeding preference may be plastic. In particular, host generalist species are of medical veterinary importance because they are more likely responsible for pathogen spillover into human and other animal species of economic and conservation concern. Although information on blood feeding preferences across the globe have accumulated during the last 50 years, there is not a clear understanding on how human induced land use changes affect those host preferences. Thus, here I conducted a meta-analysis summarising data since 1966 to the present. Results indicate that in general the Culicidae feed less on birds and wild mammals in urban environments, with a gradual increase on humans going from non-urban to urban conditions. Moreover, mosquito feeding rate on domestic mammals' peaks at periurban conditions. The analysis demonstrates that there is a significant publication bias, where most reported species are underrepresented and more knowledge is provided for a handful of species with medical veterinary importance. Blood meals from *Culex quinquefasciatus* and *Culex pipiens* were rarely reported in non-urban areas, they rather fed on birds and humans at the periurban and urban conditions. In the case of the highly invasive tiger mosquito (*Aedes albopictus*), feeding rate on humans, domestic mammals, and birds was significant in the periurban condition, suggesting that this mosquito species is very important across the human-domestic-wild animal interface. Hence, the analysis suggests that mosquitoes readily feed on humans when available and that special attention must be placed across periurban areas (i.e. rural agricultural and sub-urban areas), where mosquito diet is more diverse in terms of vertebrate groups, and pathogen spillover may be more likely.

Keywords: blood meal, Culicidae, landscape parasitology, urban parasitology, mosquito feeding behaviour, conservation medicine, One Health

Introduction

Human activities are destroying and modifying natural environments at an unprecedented rate, mostly via urbanisation and agriculture to fulfil modern human needs (Adler and Tanner 2013). Yet, those environmental modifications that are beneficial to humans are having unintended and detrimental consequences for biodiversity and the functioning of ecosystems (Dirzo *et al.* 2014, Maxwell *et al.* 2016), to such a degree that a new geologic epoch named the Anthropocene has been established (Waters *et al.* 2016). Moreover, there are also negative impacts on human health itself due to air, soil, and water pollution, causing both infectious and non-infectious diseases in urban and periurban areas (e.g. Clark and Soares Magalhães 2018, Rothenburger *et al.* 2018).

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Anthropogenic activities are bringing closer humans to wildlife through narrowing the gap of the human-domestic-wild animal interface (Hassell *et al.* 2017). This opens the door to parasite spillovers among hosts that would rarely meet under well-conserved ecosystems, subsequently leading to emergent and re-emergent diseases (Allen *et al.* 2017). Thus, it is a medical and veterinary health priority to understand how human activities modify host-parasite ecological interactions (e.g. Hossack *et al.* 2013, Pearson *et al.* 2016, Van Hoesel *et al.* 2019).

Human emergent disease events have increased through time; approximately 60% of those events derive from animals (i.e. they are zoonotic in origin) and about three quarters of those come from wild animals (Jones *et al.* 2008). A recent study on human emergent diseases identified global risk factors for spillovers, where emergent events are more likely in areas with strong land use changes toward agricultural practices, in regions with humid and warm climate (i.e. tropics), in regions with larger urban land surface area, and in regions with higher mammal diversity (Allen *et al.* 2017). In this sense, it is interesting to note that although cities just cover approximately 3% of the planet´s surface area (Liu *et al.* 2014), their impacts extend well beyond city borders onto the surrounding periurban and natural environments, having impacts that can range from 10 to more than 100 times the size of a city (Adler and Tanner 2013, Wigginton *et al.* 2016). Considering that by 2025 circa 65% of the human population will live in cities, we can expect an increase of spillover zoonosis into human populations, particularly at periurban and rural productive zones where the human-domestic-wild animal interface is narrowest.

Of the human emergent diseases, around 15% are vector-borne (Jones *et al.* 2008), which means that we must aim to understand the blood feeding preferences of arthropods to predict and manage health hazards and risks (Hosseini *et al.* 2017, Robles-Fernández *et al.* 2021). Human activities impact the diversity and structure of arthropod vectors, which can range from modification of species abundances without major impacts on richness (e.g. Abella-Medrano *et al.* 2015, Ferreira *et al.* 2016), to a reduction on species richness and dominance by one or a few species in more urbanised areas, favouring non-native invasive species (e.g. *Aedes albopictus*, *Culex quinquefasciatus*; Juliano and Lounibos 2005, Martínez-de la Puente *et al.* 2016, Talbot *et al.* 2019). Among arthropods, the Diptera is an insect order of medical importance, and within this order the family Culicidae (i.e. mosquitoes) includes some of the most important vector species of human diseases (e.g. dengue virus [DENV], yellow fever virus [YFV], malaria; Sotomayor-Bonilla *et al.* 2019, Wolfe *et al.* 2007). Extrinsic and intrinsic factors (e.g. odours, humidity, gender, genetics, host density) affect mosquitoes' feeding preferences, and depending on local conditions preferences can be highly plastic (Takken and Verhulst 2013). Yet, to my knowledge, there has been no attempt to synthesise how land use type may affect mosquito feeding preferences.

The Order Diptera is one of the most diverse among the insects accounting to about 159,000 species worldwide, included in circa 158 families (Ibáñez-Bernal *et al.* 2020). The family Culicidae is included in the infraorder Culicomorpha and the superfamily Culicoidea (Borkent 2012). It includes approximately 3,563 described species worldwide, which are included in 41 genera; there are two recognised subfamilies, Culicinae and Anophelinae, both of which include blood feeding species (Ibáñez-Bernal *et al.* 2020). The Culicidae is particularly diverse in tropical regions, and many species have been introduced to new geographical locations via human transport, where they have invaded and negatively affected local populations of both wild animals and humans acting as competent vectors of a diverse array of pathogens (e.g. avian malaria in Hawaii; van Riper *et al.* 1986; DENV in the Americas; Dick *et al.* 2012, Messina *et al.* 2014), and also as competitors for native mosquito species (Juliano and Lounibos 2005). In this manuscript we aim to respond the question: is there a change in mosquito feeding preferences toward humans and their domestic animals as a function of anthropogenic land-use change? Previous studies have shown that many mosquito species have plastic feeding preferences, modulating their choice to whatever host is available and more abundant (e.g. Jansen *et al.* 2009, Lefèvre *et al.* 2009, Santiago-Alarcon *et al.* 2012, Takken and Verhulst 2013). Thus, we expected to find an interaction effect between host type and habitat condition, with an increase of human and domestic mammal feedings in periurban areas which is where the human-domestic-wildlife interface is narrowest.

Methodology

Search strategy, selection of papers and study inclusion criteria

The study criteria for inclusion in the meta-analysis were (1) that each manuscript had information for the type of host (see below) detected in blood meals with no bias in identification method (i.e. that the methods to determine hosts are not aimed to specific species, but rather to detect any vertebrate blood source), (2) that the variable habitat type can be assigned to at least one of three categories (see below) for the blood meals with host determination, and (3) that it was a field study (i.e. no experiments and/or baited traps).

Papers were initially selected based on the title, key words, abstract and making sure that they reported a table, or at least a frequency graph, that included the number of times a mosquito species fed on a specific host.

We followed three search strategies based on recommendations by Gurevitch and Nakagawa (2015):

1. Search in Web of Science (all databases) from 1966 to present.

TS = ((Mosquito* OR Culicidae OR Culex OR Aedes OR Anopheles OR Wyeomyia OR Uranotaenia OR Coquillettidia OR Mansonia OR Sabethes OR 'mosquito vector') AND ('blood meal' OR 'feeding preference' OR 'host breadth' OR 'host preference' OR 'host choice') AND ('land use*' OR 'urban*'))

Results: 158

2. Google search from 1950 to 2020. I reviewed the first 400 hits (100 more than those recommended by Haddaway *et al.* 2015).

(mosquito OR Culicidae) AND (blood meal OR feeding preference) AND (land use OR urban)

3. I also used forward and backward searchers, mostly focusing on earlier reviews related to the research topic. In particular, syntheses by Hocking (1971), Tempelis (1975), Washino and Tempelis (1983), Takken and Verhulst (2013), and Walton and Reisen (2014).

After the first screening we kept 123 papers of those retrieved from the tree steps described above. Finally, after a thorough screening we retained 59 papers that fulfilled our inclusion criteria. The reference of the papers with their matching IDs used for analyses are listed in Table 1.

Table 1. List of studies included in analyses with their matching ID and the country where the study was conducted.

ID	Reference	Country	ID	Reference	Country
1	Jansen *et al.* (2009)	Australia	31	MacKay *et al.* (2010)	USA
2	Cohen *et al.* (2009)	USA	32	Brugman (2016)	UK
3	Pappa *et al.* (2011)	Equatorial Guinea	33	Bataille *et al.* (2012)	Ecuador
4	Estep *et al.* (2011)	USA	34	Kading *et al.* (2013)	Guatemala
5	Rath *et al.* (2015)	India	35	Blosser *et al.* (2016)	USA
6	Greenberg *et al.* (2012)	USA	36	Kay *et al.* (1985)	Australia
7	Loyola *et al.* (1993)	Mexico	37	Kim *et al.* (2017)	Korea
8	Tedrow *et al.* (2019)	Madagascar	38	Richards *et al.* (2006)	USA
9	Kamau *et al.* (2003)	Kenya	39	Valerio *et al.* (2010)	Italy
10	Asigau *et al.* (2019)	Ecuador	40	McPhatter *et al.* (2017)	USA
11	Hassan *et al.* (2003)	USA	41	Edman (1971)	USA
12	Wekesa *et al.* (1997)	USA	42	Edman (1974)	USA
13	Burkett-Cadena *et al.* (2008)	USA	43	Muñoz *et al.* (2011)	Spain
14	Yewhalaw *et al.* (2014)	Ethiopia	44	Campbell *et al.* (2013)	USA
15	De Carvalho *et al.* (2014)	Brazil	45	Kim *et al.* (2009)	Japan
16	Guo *et al.* (2014)	China	46	Kay *et al.* (2007)	Australia
17	Ritchie and Rowley (1981)	USA	47	Van den Hurk *et al.* (2003)	Australia
18	Abella-Medrano *et al.* (2018)	Mexico	48	Hoyer *et al.* (2019)	USA
19	Downe (1960)	Canada	49	Pereira dos Santos *et al.* (2018)	Brazil
20	Zimmerman *et al.* (2006)	Brazil	50	Forattini *et al.* (1987)	Brazil
21	Kim *et al.* (2009)	Japan	51	Forattini *et al.* (1989)	Brazil
22	Hall-Mendelin *et al.* (2012)	Australia	52	Goodman *et al.* (2018)	USA
23	Hamer *et al.* (2008)	USA	53	Flies *et al.* (2016)	Australia
24	Sousa *et al.* (2001)	Sao Tome	54	Kent *et al.* (2009)	USA
25	Tempelis and Reeves (1964)	USA	55	Osório *et al.* (2013)	Portugal
26	Kek *et al.* (2014)	Singapore	56	Snow and Boreham (1973)	Gambia
27	Cupp and Stokes (1976)	USA	57	Muturi *et al.* (2008)	Kenya
28	Muñoz *et al.* (2012)	Spain	58	Omondi *et al.* (2015)	Kenya
29	Martha *et al.* (2015)	Uganda	59	Stenn *et al.* (2018)	USA
30	Olson *et al.* (2020)	USA			

Statistical analysis and data extraction

The response variable for analysis is the number of blood meals (i.e. a count variable, Poisson distribution) taken by mosquitoes, and the covariates or moderators included the categorical variables habitat type (i.e. urban, peri-urban, non-urban) and host species (i.e. human, non-human domestic mammal, non-human wild mammal, bird, reptile, amphibian). Urban is defined as a city area where houses are constructed of concrete or other solid materials, there are paved streets and highways, also all necessary health and education services, as well as other amenities. Peri-urban was defined as areas of human habitation (e.g. villages, ranches), where a health service is available for the community but houses and roads are not necessarily made of solid materials. Moreover, peri-urban human habitations commonly have farm animals (e.g. cows, horses, fowl) or include agricultural lands. Finally, non-urban was defined as a natural area (e.g. a forest reserve), isolated from human settlements but that it may be nearby a ranch. Regarding blood meal identification, categories to classify hosts included human, domestic mammal, wild mammal, bird and reptile. Mixed blood meals were considered whenever the study reported enough information to include the sample in one or more of the considered categories. I did not consider studies that only used landing information to determine host preferences, but they were considered for discussion purposes.

I used the escalc() function from the 'metaphor' package for R (Viechtbauer *et al.* 2010) to calculate the observed effects and corresponding sampling variances of the event counts (i.e. number of blood meals: escalc(measure = 'IRLN', xi = blood meals, ti = sample size, data = data), where IRLN is the log transformed incidence rate). Subsequently, considering the non-independence of the retrieved data (i.e. observed effects), I used the rma.mv() from the 'metaphor' package to perform random-effects models due to possible clustering in the data (i.e. differences across studies outcomes due to variations in trapping methods, geographic region, and also mosquito species, along with unidentified characteristics of study settings and procedures). For analyses, I filtered the initial data base containing 928 observations to remove studies with a sample size lower than 10 and with blood meal records of less than 5; the final data set included 524 observations. I used the Q statistic to measure heterogeneity among study outcomes. I further used the partition of the Q statistic into its components Q_M (i.e. heterogenetity accounted for by covariates) and Q_E (i.e. heterogeneity unaccounted by analysed covariates; Gurevitch and Nakagawa 2015). I tested for publication bias using funnel plot asymmetries along with Kendall's rank correlation tests. Finally, we used Cook's distances and DFbetas to detect potential studies influencing analyses (Viechtbauer *et al.* 2010). The above-described procedures were performed for all Culicidae, and for the species *Cx. quinquefasciatus* (here we also included *Cx. pipiens*), and *Ae. albopictus* separately as they had enough studies and observations, and also due to their widespread distribution and medical importance.

Results

In general, mosquitoes reduce their feeding on birds and wild mammals in the urban environment. In contrast, mosquitoes' blood meals on humans increase gradually from non-urban to urban environments, and the feeding rate on domestic mammals peaks at periurban conditions (Table 2, Figure 1). Results demonstrate that there is a strong interaction between sources of blood meals and habitat condition, where each host group nested within the habitat condition explains ~16% of the variance (see variance components in Table 2). Yet, model's results must be interpreted cautiously because there was a large variation not accounted for by predictor variables (i.e. Q_E, see Table 2). The funnel plot and Kendall's test demonstrate publication bias in the sense that for most of the mosquito species' feeding preferences are virtually unknown, and thus, they are underrepresented across studies (see inset in Figure 1). Although Cook's distances showed that the study by Kay *et al.* (1985) had an influence on the analysis given its large sample size, the effect of such study was not consistent for the interacting moderators in the model according to DFBETAS results (see Supplementary Figure A1).

Cx. quinquefasciatus and *Cx. pipiens*. These two species did not commonly feed on domestic mammals, and their blood meals were rarely recorded in the non-urban condition. Instead, they commonly fed on wild mammals at the periurban condition and on birds and humans at both the periurban and urban conditions. There was a feeding rate increase on humans going from the periurban to the urban condition (Table 3, Figure 2). Results indicate that these two species seem to prefer areas perturbed by human activities. Q statistics showed that there was variance

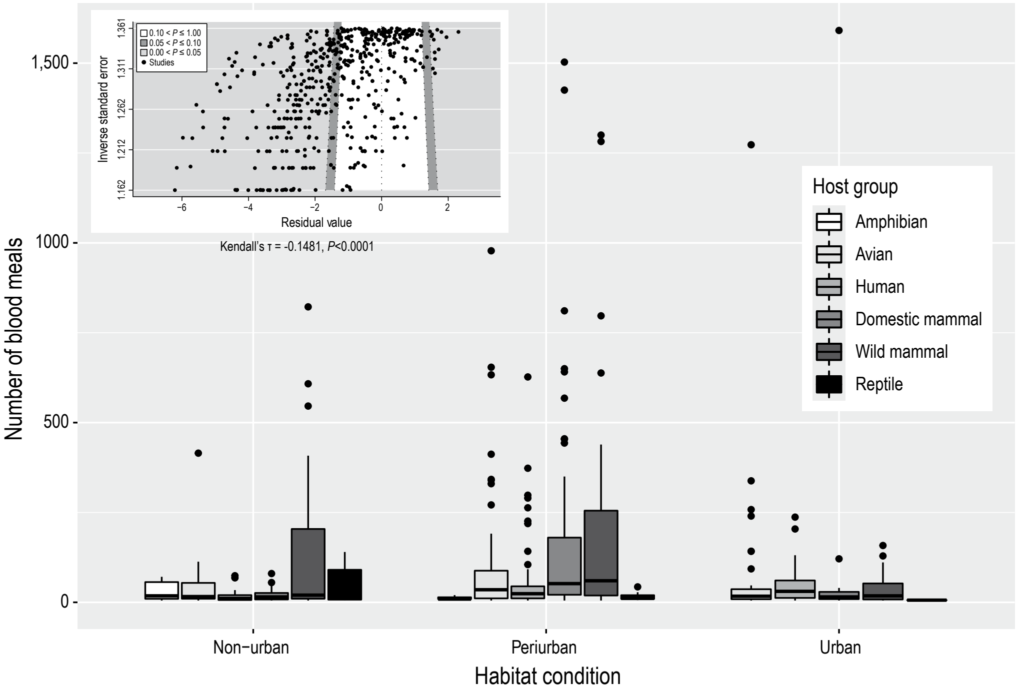

Figure 1. Number of blood meals recorded for Culicidae *as a function of habitat condition and vertebrate host group. Inset shows the funnel plot and the Kendall's tau test for publication bias.*

not accounted for by the moderators (Table 3). There was not publication bias according to the funnel plot and Kendall's test (see inset in Figure 2), but Kendall's had a non-significant trend, so publication bias and influence by some studies is likely. Cook's distances showed that the studies by Kay *et al.* (1985), Forattini *et al.* (1987), and Mackay *et al.* (2010) had an influence on the model, but such influence was not homogeneous across moderators; other studies (e.g. Guo *et al.* 2014) influenced some of the moderators (see DFBETAS results in Supplementary Figure A2).

Aedes albopictus. Most blood meals for this mosquito species were recorded in periurban and urban environments, mostly feeding on mammals and birds (Figure 3). Feeding rate on humans and domestic mammals was significantly larger at the periurban condition, where this mosquito also readily fed on birds (Table 4, Figure 3). Results suggest that this mosquito species is important at the human-domestic-wildlife interface, and it also feeds on amphibians and reptiles. *Q* statistics showed that there was variance not accounted for by the moderators (Table 4), but there was not bias according to the funnel plot and no publication bias according to Kendall's test (see inset in Figure 3). Cook's distances and DFBETAS showed that the study by Goodman *et al.* (2018) had an influence on the model that was homogeneous across moderators (Supplementary Figure A3).

Table 2. Results from the mixed-effects meta-analysis model for the response variable blood meals (log-transformed incidence rate) as a function of habitat type and host group.[1,2]

Variable	Estimate	SE	z	*P*-value	95% CI
Intercept	-2.95	0.57	-5.09	<0.0001	-4.08/-1.81
Urban	-2.65	0.89	-2.95	0.003	-4.4//0.89
Avian	1.82	0.56	3.20	<0.002	0.70/2.94
Wild mammal	1.37	0.56	2.40	0.016	0.25/2.48
Urban × Avian	2.80	0.89	3.12	<0.002	1.04/4.56
Periurban × Human	1.76	0.81	2.16	0.03	0.16/ .36
Urban × Human	3.89	0.89	4.33	<0.0001	2.13/5.65
Periurban × Domestic mammal	1.70	0.81	2.09	0.036	0.11/3.29
Urban × Domestic mammal	3.66	0.89	4.08	<0.0001	1.90/5.42
Urban × Wild mammal	2.84	0.89	3.16	<0.002	1.08/4.60
Q statistics:	Q_M=7080.56, df=16, P<0.001; Q_E=62709.3, df=507, P<0.001				

Variance components

	Estimate	√	Levels	Factor
σ^2	0.54	0.74	58	ID or study
τ^2_1	0.32	0.56	11	amphibian
τ^2_2	0.31	0.56	135	bird
τ^2_3	0.31	0.56	121	human
τ^2_4	0.31	0.56	148	domestic mammal
τ^2_5	0.32	0.56	92	wild mammal
τ^2_6	0.31	0.56	17	reptile
ρ	0.50			

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[1] Random factor included the study (~1|ID) and a group (i.e. ~host group|habitat type) component. This analysis includes all the 59 Culicidae studies that met our inclusion criteria. Only significant interactions are shown.

Discussion

The mosquitoes (Diptera: Culicidae) is a speciose family that includes many species of medical and veterinary relevance and that are distributed worldwide with the exception of Antarctica (Borkent 2012, Ibáñez-Bernal *et al.* 2020). They are known to transmit some of the most serious human diseases, such as malaria, filariasis, and several species of the *Flaviviridae* (Santiago-Alarcon *et al.* 2012, Sotomayor-Bonilla *et al.* 2019). Most of those pathogens have a wild zoonotic origin (Jones *et al.* 2008), which means that the mosquito vectors must have acted as bridges across different species that may or may not be closely related (e.g. *Culex territans* feeds on all terrestrial vertebrate groups; Burkett-Cadena *et al.* 2008; see also Hamer *et al.* 2008). Thus, it is of outmost importance to determine the blood feeding preferences of mosquitoes. Results of this paper indicate that the Culicidae is composed of species that have flexible blood feeding preferences, and that different land use types affect mosquitoes' host selection (see also Adler *et al.* 2011, Takken and Verhulst 2013). As expected, mosquitoes increase their feeding on humans as anthropogenic influence intensifies, and their feeding on domestic animals reaches a maximum at periurban conditions (i.e. rural agricultural areas, villages close to natural habitats, production systems in the outskirts

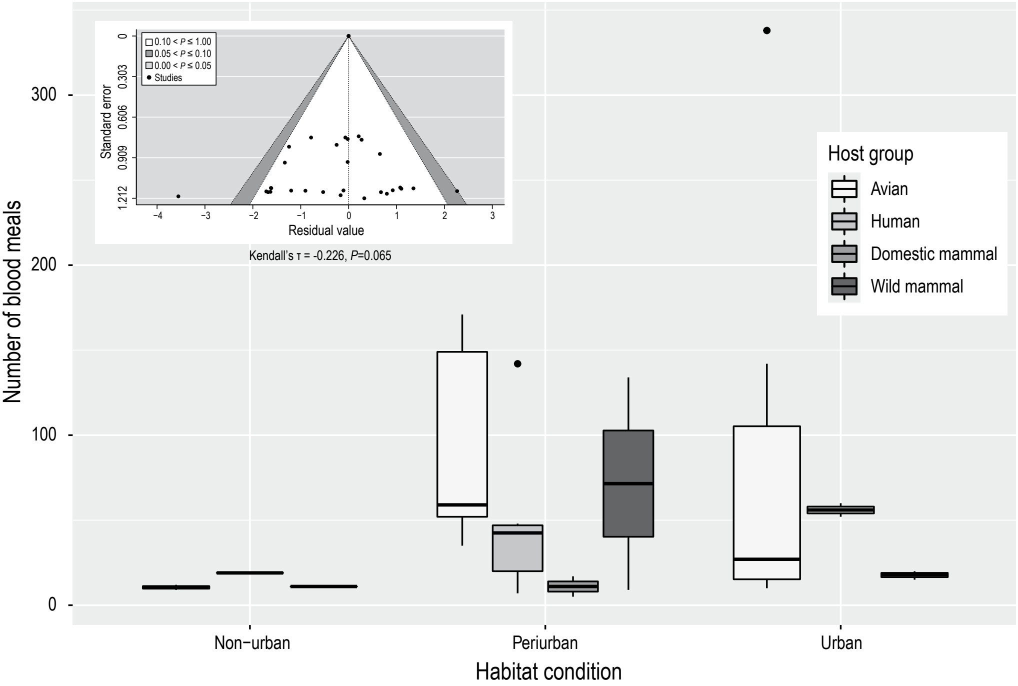

Figure 2. Number of blood meals recorded for Culex quinquefasciatus *and* Cx. pipiens *as a function of habitat condition and vertebrate host group. Inset shows the funnel plot and the Kendall's tau test for publication bias.*

Table 3. Results from the mixed-effects meta-analysis model for the response variable blood meals (log-transformed incidence rate) as a function of habitat type and host group for the species Culex quinquefasciatus.[1]

Variable	Estimate	SE	z	*P*-value	95% CI
Intercept	-3.67	1.31	-2.79	<0.006	-6.24/-1.09
Domestic mammal	-2.83	1.18	-2.40	0.016	-5.15/-0.52
Periurban × Human	-0.89	0.38	-2.32	0.02	-1.64/-0.14
Urban × Human	-2.32	0.39	-5.84	<0.001	-3.10/-1.54
Q statistics:	Q_M=308.1, df=14, *P*<0.001; Q_E=2088.5, df=18, *P*<0.001				
Variance components					
	Estimate	√	Levels	Factor	
σ^2	1.64	1.28	24	ID or study	

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[1] Random factor included the study (~1|ID) variable. This analysis includes 33 studies that met our inclusion criteria. Only significant interactions are shown.

[2] CI = confidence interval; df = degree of freedom; SE = standard error.

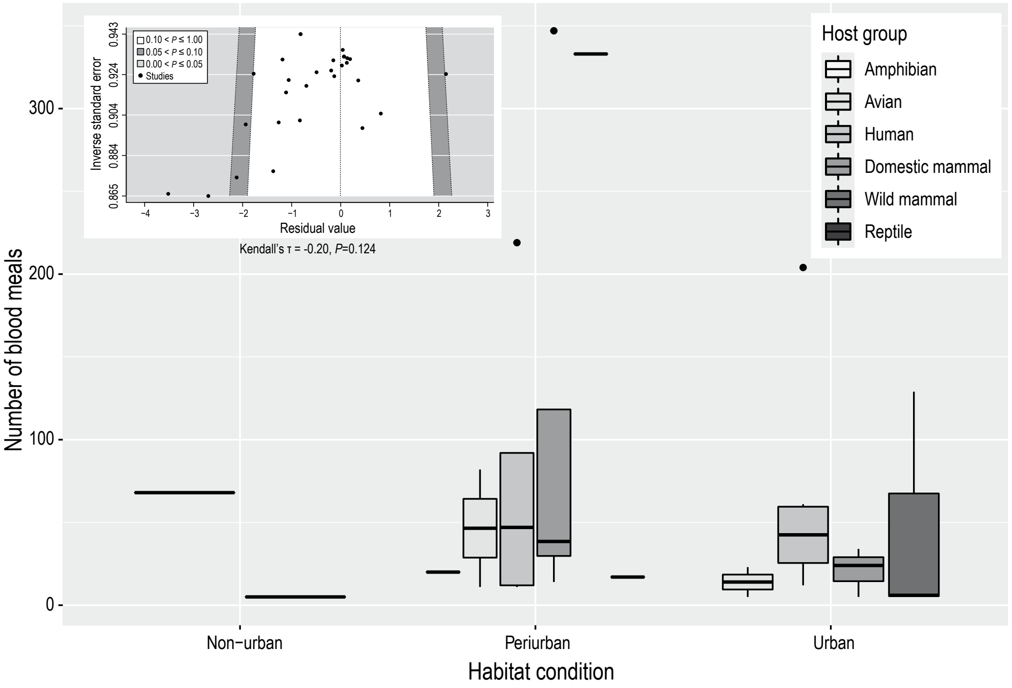

Figure 3. Number of blood meals recorded for Aedes albopictus *as a function of habitat condition and vertebrate host group. Inset shows the funnel plot and the Kendall's tau test for publication bias.*

Table 4. Results from the mixed-effects meta-analysis model for the response variable blood meals (log-transformed incidence rate) as a function of habitat type and host group for the species Aedes albopictus.[1,2]

Variable	Estimate	SE	z	*P*-value	95% CI
Intercept	-4.75	0.81	-5.86	<0.001	-6.34/-3.16
Urban	1.42	0.51	2.77	<0.006	0.41/2.42
Avian	0.82	0.35	2.34	0.019	0.13/1.51
Human	3.42	0.54	6.26	<0.001	2.35/4.49
Domestic mammal	0.81	0.28	2.81	<0.005	0.24/1.38
Wild mammal	2.81	0.23	12.21	<0.001	2.36/3.26
Urban × Human	-1.13	0.48	-2.34	0.019	-2.08/-0.18
Periurban × Domestic mammal	1.91	0.19	10.05	<0.001	1.54/2.28
Q statistics:	Q_M=731.82, df=11, P<0.001; Q_E=618.60, df=18, P<0.001				

Variance components

	Estimate	√	Levels	Factor
σ^2_1	0.28	0.53	10	ID or study
σ^2_2	1.21	1.10	4	Continent

of cities). Thus, the risk of pathogen spillover into humans must be highest at periurban conditions where the human-domestic-wildlife interface is narrowest (Hassell *et al.* 2017). Nonetheless, some caveats are important here; first, the meta-analysis showed a strong publication bias, most of the studies are focused on a few mosquito species of medico veterinary importance. This is expected as usually there are more research funds to investigate issues of direct impact on human health and economy. Second, results demonstrate that our knowledge on the blood feeding preferences of most mosquito species is at best precarious, which would limit our ability to determine mosquito-borne pathogen sources or reservoirs. It is important to notice that among the Diptera, the family Culicidae is the best known mostly due to their human impacts. This signals another problem for researchers, knowledge on other Diptera families involved in parasite transmission is even more limited than for Culicidae, leaving a huge gap of knowledge from where unknown lurking pathogens may be awaiting their chance to jump into novel hosts, not just humans. Thus, a lot more research efforts must be directed into understanding Diptera feeding patterns, without restricting detection to a few hosts as is the case for many of the studies not included in the meta-analysis (i.e. focused on humans and domestic animals; see below and Additional references in the Supplementary Materials). The recommendation is to plan studies where all groups of vertebrates are considered as potential feeding sources, and where anthropogenic impacts (i.e. land use types) are explicitly considered in studies' designs.

Only four species (*Cx. quinquefasciatus*, *Ae. albopictus*, *Cx. pipiens*, and *Culex annulirostris*) had more than 30 records in our data base, confirming the bias knowledge we possess on blood feeding preferences. From those, we analysed together *Cx. quinquefasciatus* and *Cx. pipiens* because their taxonomic definition according to some dipterologists is still not quite settled and sometimes they are placed together as sub-species. Regardless of their taxonomy, they are implicated in the transmission of important human pathogens (e.g. *Wuchereria bancrofti*, Japanese encephalitis virus, Saint Louis encephalitis virus; Sotomayor-Bonilla *et al.* 2019), and also pathogens of veterinary and conservation concern (e.g. West Nile virus, avian malaria; Reiter and LaPointe 2009). Also important is to consider that these *Culex* species along with *Ae. albopictus* are non-native highly invasive mosquitoes that have decimated or drastically affected native vertebrates (e.g. Hawaiian endemic birds; van Riper *et al.* 1986) and that are able to outcompete native mosquito species (Juliano and Lounibos 2005). In particular, the tiger mosquito (i.e. *Ae. albopictus*) is currently expanding its distribution (Lowe *et al.* 2000; Bonizzoni *et al.* 2013). The tiger mosquito is native to Southeast Asia and was considered a non-urban species inhabiting forest edges, but during the last decades has expanded globally and it is currently well established in urban environments and it is considered a public health threat (Ferraguti *et al.* 2020, Muñoz *et al.* 2011). Results from the meta-analyses suggest that these mosquito species are well adapted to urban and periurban settings, and that they have generalist feeding behaviour, taking blood meals from avian and mammal hosts (see also Martínez-de la Puente *et al.* 2016, Takken and Verhulst 2013). The results also indicate that the *Culex* species gradually increase their feeding rate on humans from non-urban to urban environments, and for the tiger mosquito its feeding on humans and domestic mammals is highest in the periurban condition. Finally, data for *Cx. annulirostris* came only from Australia, and it indicated that this species is also well adapted to habitats with anthropogenic impacts (no blood meals from non-urban conditions were recorded according to our habitat definitions, see methods), but unlike the others it seems to feed more commonly on non-human mammals and birds and its diet also includes reptiles (Supplementary Figure A4). Although human activities are clearly aiding the expansion of invasive mosquito species (e.g. Ferraguti *et al.* 2016) and altering parasite community dynamics and structure (e.g. Hernández-Lara *et al.* 2017, 2020), research efforts must be directed to whole communities of blood sucking Diptera and their hosts (e.g. Ferraguti *et al.* 2018) otherwise we are poised for new emergent surprises in the near future.

Within cities, zoos and greenspaces are special places in the urban environment, because they add habitat heterogeneity and provide a variety of blood sources that mosquito vectors are not able to find either in heavily built areas or in the surrounding natural environment (Chagas *et al.* 2017). Such greenery within cities may allow some mosquito species to persist which otherwise would disappear from urban settings (i.e. urban avoiders and urban utilisers; *sensu* Fischer *et al.* 2015). In particular, lack of proper veterinary control of zoo animals and surveillance of wild animals within the zoo environment may permit pathogen spillover in several directions: from wild animals to humans and zoo animals, from zoo animals to humans and native wild animals, and from humans to animals (in particular primates). Thus, zoos must have permanent surveillance strategies to monitor arthropods of medical and veterinary importance (Adler *et al.* 2011). Furthermore, studies analysing the vector community structure along non-urban–urban gradients will help to determine species that are filtered at the urban border, which ones are able to persist and to adapt, and to study their blood feeding preferences (e.g. Abella-Medrano *et al.* 2015, 2018). Therefore, integrative studies at the assemblage level are urgently needed (e.g. Ferraguti *et al.* 2018, Van Hoesel *et al.* 2020).

Finally, many of the retrieved papers were left out of the analysis because they either used serological tests aimed at specific host species or because it was not possible to assign identified blood meals to a habitat type (see Additional references in the Supplementary Materials). Most of the studies using serological tests were from the early 90's or earlier, and they were aimed at specific hosts (particularly humans and non-human domestic mammals) as part of surveillance efforts. The only serological studies considered in the study were those using anti-sera for all terrestrial vertebrate groups, including amphibians. There were only five cases in which I was not able to assign each blood meal to a specific land use type as defined in this study, even when those studies had samples from different habitat types (i.e. Börstler *et al.* 2016, Martínez-de la Puente *et al.* 2016, Rizzoli *et al.* 2015, Sawabe *et al.* 2010, Shahhosseini *et al.* 2018). Thus, it is highly recommended that authors analysing mosquito blood meals assign each sample to its corresponding habitat type, which also implies a detailed description of each study site in the methods sections. Moreover, whenever possible authors should aim to use PCR techniques with primers that would amplify any vertebrate order including fishes (e.g. Kim *et al.* 2017, Miyake *et al.* 2019). If using serological tests, they should aim to cover all vertebrate orders as well. Taken all together, we must aim at integrative comprehensive vector studies at the assemblage level and across perturbation gradients whenever resources allow; only then, we will acquire enough information on vector blood feeding preferences and ecological dynamics that will permit better gauging pathogen hazards and risks.

Supplementary materials

Supplementary material can be found online at https://doi.org/10.3920/978-90-8686-9312_9

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10. Surveillance of invasive mosquito species in islands with focus on potential vectors of zoonotic diseases

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Abstract

The invasive species (IS) introduced in islands cause important impacts due to the vulnerability of their ecosystems. The invasive potential of certain mosquito species and their role as vectors of pathogens is one of the main concerns for public and animal health. The introduction of IS such as *Aedes albopictus* (Skuse 1895), *Aedes aegypti* (Linnaeus 1762) and *Culex quinquefasciatus* Say 1823 are also related to outbreaks of vector-borne diseases (VBD), such as yellow fever, dengue and Zika. Here, we review the surveillance activities on mosquito IS conducted in several islands of different origin (i.e. volcanic vs continental origin) located in different countries of the world. Those countries included Cabo Verde, Greece, Italy, Portugal, Spain and the USA. In regards to continental islands, *Ae. albopictus* was detected in the Balearic Islands (Spain) in 2012 despite monitoring at points of entry lead by national authorities since 2008. Greece comprises over 6,000 islands and islets with first record of *Ae. albopictus* in Corfu in 2003. In Italy, *Ae. albopictus* was first detected in Sicily in 2004 where several cases of filariasis by *Dirofilaria repens* in dogs and humans have been reported. Volcanic origin islands are characterised by having all mosquito fauna introduced from the continent. In Cabo Verde, *Anopheles arabiensis* is the main vector of malaria and can also transmit lymphatic filariasis. *Ae. aegypti* is also present in Cabo Verde since 1930 causing several outbreaks of dengue and Zika in 2009 and 2015. In Spain, *Ae. aegypti* was detected in Fuerteventura (Canary Islands) in 2017, but the fast intervention of local authorities reached its eradication in 2019. In Portugal, *Ae. aegypti* was first recorded in Madeira in 2006 with a single outbreak of dengue in 2012. In the USA, the islands of Hawaii have currently six established IS of mosquitoes including the four top vector species *Ae. albopictus*, *Ae. aegypti*, *Aedes japonicus* and *Cx. quinquefasciatus*, which have been implicated in outbreaks of dengue and transmission of *Dirofilaria immitis* and *Plasmodium relictum*.

Keywords: *Aedes*, *Culex*, Dengue, *Dirofilaria*, insecticide resistance, monitoring, mosquito invasive species

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Introduction

Invasive species (IS) are those species introduced frequently by human means outside their native distribution area then causing significant negative impacts on the environment, agronomy, public health, economy and culture. Island ecosystems are highly vulnerable than the continental ones. Hence, the introduction of IS has important effects on islands, since they may destabilise island ecological communities, including impacts on endemic fauna and flora. In fact, this impact could be amplified by climate change, urban development, etc. (Russell *et al.* 2017).

During the last decades, several mosquito species of genus *Aedes* have been introduced in different countries worldwide. The invasive potential of this species and their role in the transmission of vector-borne diseases (VBDs) is one of the major concerns for public health. The Asian tiger mosquito *Aedes albopictus* (Skuse 1895), the yellow fever mosquito *Aedes aegypti* (Linnaeus 1762) and the southern house mosquito *Culex quinquefasciatus* Say 1823 are examples of invasive mosquitos in Europe and the Americas (Juliano and Philip-Lounibos 2005, Kraemer *et al.* 2015). In fact, the Global Register of Introduced and Invasive Species (GRIIS) considers *Cx. quinquefasciatus* as one of the top ten most globally prevalent invasive species on islands.

The introduction of the aforementioned IS are commonly related to transport and trade and their geographical distribution overlaps with the spread of several arboviruses i.e. yellow fever (YFV), dengue (DENV), Chikungunya (CHIKV) and Zika (ZIKV) (Kraemer *et al.* 2019). Other mosquito IS such as *Aedes japonicus* (Theobald 1901) and *Aedes koreicus* (Edwards 1917) are also expanding their distribution areas having stablished populations in Europe (ECDC 2020b).

The eradication of mosquito IS is challenging due to their fast-biological cycle and rapid spread. Eradication attempts are likely to be successful at the beginning of the invasion. In 1995, *Ae. albopictus* was successfully eradicated in Sardinia (Italy) due to the fast intervention of local health authorities (Nuvoli and Pantaleoni 2003) but the species was recorded again in 2006 (Cristo *et al.* 2006) and is considered currently stablished (ECDC 2020b). The continue incursions of *Ae. albopictus* through transport of goods and vehicles hinder definitive eradication.

Information regarding the introduction of mosquito IS on islands in Spain, Portugal, Cabo Verde, Italy, Greece and USA is summarised in the current chapter, focusing on surveillance and control methods. The islands have been categorised as non-volcanic islands, where fauna have continental origin, then ancient origin, and volcanic islands, where the fauna is considered as 'recently' established.

Non-volcanic islands

The invasive mosquito **Aedes albopictus** *in the Balearic Islands*

The Balearic Islands archipelago is located in the southwestern part of mainland Spain (western Mediterranean; 39°30'N 3°00'E). The archipelago comprises four major islands: Mallorca, Menorca, Ibiza and Formentera, the uninhabited National Park of Cabrera Island and other surrounding islets. Majorca is the largest island (3,640 km^2) with 896,000 inhabitants in 2019 and 16.5 million visitors (mainly tourists) during high season in 2019 (IBESTAT 2020). Its climate is characterised by hot dry summers and wet winters. Mean annual temperatures in the Balearic Islands range from averages of 24.5 °C during the hottest month (August) to 10 °C during the coldest month (January). The mean annual precipitation in the island is 425 mm (AEMET 2012).

A total of 17 mosquito species are currently present in the Balearic Islands (Robert *et al.* 2019), being *Ae. albopictus* the only invasive mosquito species stablished in the archipelago. This species, which shows a strong anthropophilic behaviour (Muñoz *et al.* 2011), was first detected in Spain in Catalonia in 2004 (Aranda *et al.* 2006). Prior to that, a multidisciplinary network called EVITAR (network for the study of viruses transmitted by arthropods and rodents, Spanish Ministry of Health) conducted surveillance in used-tyres throughout 45 locations in Spain between 2003-2004 but the presence of *Ae. albopictus* was not recorded (Roiz *et al.* 2007). In fact, until 2008, there were not specific programmes for mosquito invasive species surveillance at national level.

The introduction of *Ae. albopictus* in different countries in Europe has been associated with the transport of used tyres and lucky bamboo (Collantes *et al.* 2015, Scholte *et al.* 2007). Since 2004, this species has been increasing its distribution area throughout the Mediterranean coast with evidence of dispersal by cars (Collantes *et al.* 2015, Eritja *et al.* 2017), and also in inland communities such as Basque Country and Extremadura (Bravo-Barriga *et al.* 2019; Delacour *et al.* 2015). The distribution of *Ae. albopictus* and other invasive species of mosquitos in Europe is regularly updated by the ECDC and EFSA (https://www.ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps).

In September 2012, a phone call from a resident in the municipality of Bunyola (Majorca Island, 39°39'28.89' N 2°42'4.22' E) indicated that several mosquitoes similar to *Ae. albopictus* species were seeking in the surroundings of the house. The citizen was familiarised with the species since she was originally from Argentina where *Ae. albopictus* was first detected in 1998 (Rossi *et al.* 1999). The resident sent four specimens to the laboratory of Zoology at the University of the Balearic Islands (UIB) where were identified as *Ae. albopictus* (Miquel *et al.* 2013). Afterwards, a visit to the area confirmed the presence of larval habitats in the area (barrel for water storage). From here, a GIS web viewer including a database (GIS of the mosquitoes of the Balearic Islands) was stablished by the government of the Balearic Islands (GOIB), the laboratory of Zoology (UIB) and the GIS service of the UIB. In addition, a sampling network using ovitraps was established around the initial focus to determine the level of expansion of *Ae. albopictus* in the island. At that time, the species was recorded in five municipalities: Bunyola, Calvià, Esporles, Marratxí and Palma increasing its expansion in 2013 to the municipalities of Banyalbufar and Santa Maria del Camí.

Two years after its first detection in Mallorca, in November 2014 a citizen from Montecristo municipality (Ibiza Island, 38°56'18.42' N; 01°24'56.56' E) sent two individuals of *Ae. albopictus* to the laboratory of Zoology at UIB, confirming the presence of this species in the island (Barceló *et al.* 2015). Afterwards, between July and October 2015 ten oviposition traps were placed in four hot spots of Menorca Island recording two egg-positive sites in Mahón (39°52' N 4°18'E) in September and October 2015. In addition, a citizen from Mahón sent three specimens of *Ae. albopictus* to the laboratory of Zoology at UIB, confirming the presence of this species in the island (Bengoa *et al.* 2016).

Currently, *Ae. albopictus* is considered stablished in the entire archipelago of the Balearic Islands (ECDC 2020b) but increasing its range of expansion year by year (Tavecchia *et al.* 2017). The spread of this insect from mainland Spain and between islands was probably related to the increase of commercial trade, weekly transport routes and visitors arriving by maritime transport (Barceló *et al.* 2015). For example, there is a high frequency of passengers travelling by ferry to the Balearic Islands in summer. Between Mallorca and Ibiza Island, transport routes reach 22 weekly ferries and a total of 62,655 private cars during the year of the first detection in Ibiza Island (Barceló *et al.* 2015).

Aedes albopictus is a well-known vector of medically important arboviruses, such as CHIKV, DENV and ZIKV that constitute major, global public health threats (Grard *et al.* 2014, Gutiérrez-López *et al.* 2019, Paupy *et al.* 2009). In Europe, *Ae. albopictus* is considered the vector of local transmission of DENV and CHIKV in Italy (Lazzarini *et al.* 2020, Rezza *et al.* 2007), Croatia (Gjenero-Margan *et al.* 2011) and France (Gould *et al.* 2010, La Ruche *et al.* 2010). In addition, five imported cases of ZIKV were reported in Greece during the last five years (Emmanouil *et al.* 2020). In Spain, six dengue autochthonous cases were reported for the first time in 2018 and 2019 (ECDC 2020a, Monge *et al.* 2020).

In 2005, the International Health Regulations request countries to establish vector surveillance at points of entry (ports and airports) and collect relevant data on sources of infection including vectors and reservoirs, which may lead to an international outbreak of diseases. For this, in 2008, the Spanish Ministry of Health, Consumer Affairs and Social Welfare (MHCSW), started an entomological surveillance project with two objectives: (1) an entomological surveillance in airports and ports against imported vectors of exotic infectious diseases and (2) the surveillance of potential autochthonous vectors of these diseases with special attention to the spread of *Ae. albopictus*. The airports and ports included in this surveillance belong to the communities of Madrid, Barcelona, Valencia, Zaragoza; Canary Islands and the Balearic Islands. Since 2015, it was included a new objective to assess the susceptibility of *Ae. albopictu*s against different insecticides in representative risk areas.

This project is coordinated by the Center for the Coordination of Health Alerts and Emergencies of the MHCSW and is awarded to the Department of Animal Pathology of the Faculty of Veterinary Medicine of the University of Zaragoza in collaboration with the National Microbiology Center of the Carlos III Health Institute (Laboratory of Medical Entomology). Other centers and institutions also collaborate in the project, such as the Canary Islands University Institute of Tropical Diseases and Public Health, the Baix Llobregat Mosquito Control Service in Barcelona, the Basque Institute for Agricultural Research and Development and the Faculties of Biology of the Universities of Murcia and the Balearic Islands. Since 2012, *Ae. albopictus* was the only mosquito IS recorded in the Balearic Islands. Results of surveillance in Airports and Ports of Mallorca Island with ovitraps, BG-Sentinel (BG-sentinel 2, Biogents, Germany) and CDC traps (CDC Miniature Light Trap Model 512, John W. Hock, USA) showed an increase of *Ae. albopictus* activity. The highest values were recorded in 2018, reaching an average of 228.07±142.40 eggs/ovitrap/day in the airport (Figure 1) and 14.15±9.65 adults/trap/day in the port (Figure 2).

Since *Ae. albopictus* is present in a large number of European Union countries and local transmissions have been increasing during the last decade, in 2016 the Ministry of Health of the Government of Spain has developed a National Program for Preparation and Response against VBD (MHCSW 2016), were the Autonomous and local administrations conduct surveillance and control of mosquitoes around imported cases. In the Balearic Islands, this program is coordinated by the area of public health of the Health Counselling (GOIB). This program addresses the following issues: epidemiological and entomological surveillance, vector control, protection against mosquito bites, vector control, communication, coordination and definition of risk levels.

The Balearic Islands recorded dengue cases almost every year since 2009 with a total of 42 imported cases until 2019 (Figure 3). These cases in addition to Chikungunya and Zika cases were mainly detected in travelers from South America, Thailand and Philippines (GOIB 2020). To date, no autochthonous cases have been detected in the Balearic Islands.

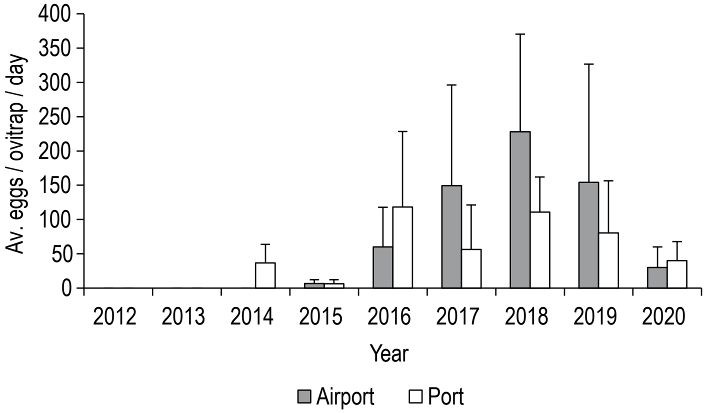

Figure 1. Number of Aedes albopictus *eggs recorded during the surveillance in ports and airports of the Balearic Islands since 2012.*

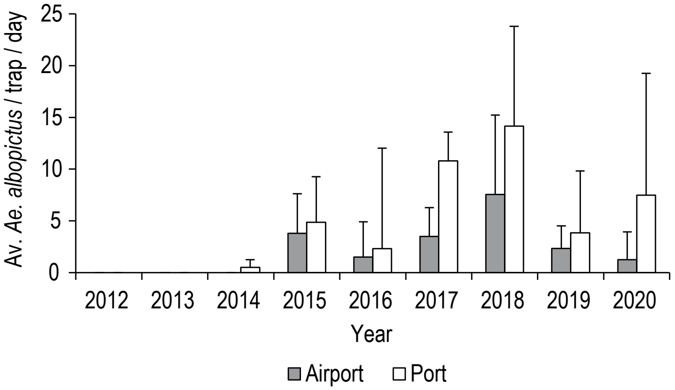

Figure 2. Abundance of Aedes albopictus *adults recorded during the surveillance in ports and airports of the Balearic Islands since 2012.*

During the beginning of the VBD surveillance program in 2016, three imported cases of Zika were detected in Calvià, Marratxí and Palma, and a single case of dengue in Puigpunyent. Inspections performed by the laboratory of Zoology (UIB) across the surroundings of the patient residences recorded activity of *Ae. albopictus* (Miranda *et al.* 2016).

In 2018, the European Cooperation in Science and Technology (COST) launched an action entitled *Aedes* Invasive Mosquitoes (AIM-COST, https://www.aedescost.eu/) with the participation of 50 institutions from 27 European Countries, including the University of the Balearic Islands. The main objective of this COST action is to establish a network of partners and institutions across Europe to address the management of the risk of introduction and spread of *Aedes* invasive species and borne viruses.

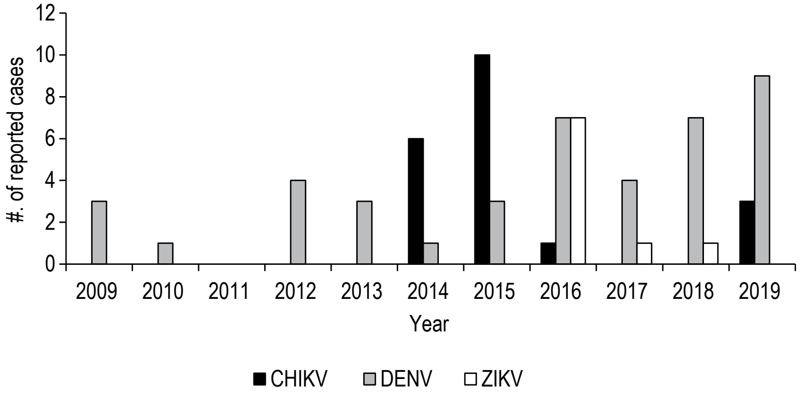

Figure 3. Number of imported cases of arboviruses reported in the Balearic Islands since 2009 (Data obtained from GOIB 2020).

Currently, the city council of each municipality of the Balearic Island is responsible of the control of *Ae. albopictus* in public areas, whereas citizens are the responsible for private zones. In fact, the presence of this species is manly associated with irrigated areas surrounding swimming pools in residential areas (Sanz-Aguilar *et al.* 2018). Hence, city council conduct campaigns to inform citizens about how to prevent the activity of *Ae. albopictus* by removing larval habitats, etc. Private companies can also play a role on this control since they can be contracted by the city council or even private institutions.

Surveying and managing invasive mosquitoes in Greece with a focus on islands

Greece has a long history of VBDs transmitted by local mosquito species, such as malaria transmitted by *Anopheles sacharovi* Favre 1903 (Spielman and D'Antonio 2001) until 1974 when the country achieved malaria free status (Danis *et al.* 2013). Another VBD historically recorded in Greece was dengue, vectored by *Ae. aegypti* (Emmanouil *et al.* 2020). After an uneventful period of low, undetected transmission of vector-borne pathogens to humans (1974-2009) a resurgence of vector-borne diseases and introductions of new pathogens have been observed in the country.

Ae. albopictus is currently the only exotic mosquito posing a significant nuisance and public health threat in Greece. The mosquito was first recorded in 2003 in the island of Corfu and Thesprotia (northwestern Greece) (Samanidou-Voyadjoglou *et al.* 2003) (presumably introduced from Italy via ferry traffic, Medlock *et al.* 2012) and was able to successfully invade the country and disperse in a little over a decade. *Ae. albopictus* populations are now established in most of continental Greece (Athens, Central Macedonia, Eastern Macedonia and Thrace, Thessaly, Peloponnese) with some of the most recent records reported from the islands of Crete and Lesvos (Patsoula *et al.* 2017). As of today, there is no evidence of autochthonous transmission of any VBDs related to *Ae. albopictus* in Greece. *Ae. albopictus* is also a competent vector of *Dirofilaria immitis* (Leidy 1856) (Diakou *et al.* 2016), yet the role of the mosquito in the local natural transmission of the filarial nematodes remains unknown. In addition, *Ae. albopictus* poses significant nuisance to the Greek populations (Kolimenakis *et al.* 2019), especially in newly invaded areas with no previous history of mosquito annoyance (Badieritakis *et al.* 2018).

The arrival of *Ae. albopictus* in Greece has spurred multiple research studies (Badicritakis *et al.* 2017, Giatropoulos *et al.* 2012, Patsoula *et al.* 2006). An EU funded project, LIFE CONOPS, has established a network of trapping stations in Greece for the surveillance of *Ae. albopictus* and for the early detection of possible newly introduced invasive mosquito species. Organised mosquito control programs in Greece are funded by the government and have been implemented in various regions of the country since 1997 (Piakis *et al.* 2007). The programs are implemented by private companies and administered by regional governments (municipality, prefecture level) who serve, also, as the supervisory authorities. The majority of the programs are based on the principles of Integrated Vector Management (IVM) (Fotakis *et al.* 2017). There are, currently, no operational vector control programs or regulatory framework targeting specifically *Ae. albopictus*.

A major issue with the intensive use of insecticides is the development of insecticide resistance. Insecticide resistance has been reported in *Ae. albopictus,* such as knockdown resistance *(kdr* mutation), yet a big knowledge gap remains relating to the susceptibility status, geographical distribution, frequency and co-occurrence of resistance traits in the vector populations (Balaska *et al.* 2020).

Greece comprises of approximately 6,000 islands and islets in the Aegean and Ionian Seas of which 227 are inhabited (EOT 2020). The climate of Greek islands is predominantly Mediterranean, with high humidity, fairly mild winters and hot, dry summers. Even though there is some recent literature available on the mosquito species for some of those islands (presence/absence studies) (Fotakis *et al.* 2020, Linton *et al.* 2007a,b) there is limited information available on the local ecology, distribution and seasonality patterns of *Ae. albopictus*. A national-level, multi-collaboration study utilised data from pest control companies, academic and research institutes and data sent by citizens in order to update distribution maps of *Ae. albopictus* (Badieritakis *et al.* 2018). The mosquito was detected across the entire country (including the Ionian islands) except of some areas of Northern Greece and the islands of the Aegean (the majority of which were not sampled). The island of Corfu, located in Ionian Sea, is where *Ae. albopictus* was recorded for the first time in Greece. Despite the increased entomological interest regarding mosquitoes of Corfu, there is limited literature to date in relation to the mosquito fauna of the island, with the most recent list of mosquito species dating back to 1937 (Stephanides 1937). The quite intense mosquito nuisance problem in Corfu (ECDC 2009), in addition to the absence of sufficient scientific data, and the increased public health risks due to the recent introduction of *Ae. albopictus* among other factors (Eckerle *et al.* 2018), create an urgent need for organising a systematic effort for the collection of entomological data for the island as well as other islands of the Ionian Sea complex.

The most recent expansion of *Ae. albopictus* in the Aegean island complex was documented in Chios, (Fotakis *et al.* 2020), where the mosquito's presence was confirmed using both morphological and molecular tools. Pyrethroid resistance mutations were also reported in *Ae. albopictus* Chios populations, as well as, other mosquito species in high frequencies despite the absence of large-scale mosquito control programs in the island. This may indicate that insecticide treatments from agriculture may be imposing strong selection pressures on the local vector populations, or that resistant alleles were pre-selected in other regions, prior to invasion.

Despite the capacity of invasive mosquitoes, and in particular *Ae. albopictus,* to spread and establish in island settings, systematic efforts to collect and monitor vector populations are seriously lacking in the majority of Greek islands. This may be attributed to difficulties in accessing those areas, in addition to the limited or absent local vector surveillance competencies (infrastructure, resources, expertise). A notable development is the creation of VectorMap-GR, an online, open access, operational management tool for entomological and epidemiological monitoring data (Fotakis 2020). VectorMap-GR was implemented for the first time in the island of Crete during 2018-2020 and has the potential to be expanded and adapted to other regional/local settings and surveillance/control efforts in Greece and neighbouring countries. The system's database currently supports the introduction and storage of data relating to mosquito monitoring, insecticide resistance, pathogen detection in vectors and sentinel animals and status of vector-borne diseases. This example could be followed by other islands or a national-level vector surveillance network that will also incorporate all island complexes, while, investing in building regional vector surveillance and control capacities.

Systematic surveillance of vectors and pathogens in vectors is the primary essential step in designing effective Integrated Vector Management programs with timely, targeted, and environmentally sensitive control strategies. Significant progress has been made in Greece towards this direction, but efforts should be reinforced and expanded across the country in a systematic and holistic approach.

Invasive mosquitoes in Sicily and its minor islands

Sicily (Italy) is the largest island in the Mediterranean basin, showing a typical Mediterranean climate, with mild winters, warm autumns/springs and hot summers. Temperatures vary among areas depending on the distance from the sea and on the presence of mountains (Blanda *et al.* 2018). The island has a high entomological interest, thanks to its high biodiversity related to the heterogeneity of bioclimatic types that range from the infra-Mediterranean to the cryo-Mediterranean type in the summit of Etna. The hydrographic component is also complex with intricate, mostly torrential, river networks (Brullo *et al.* 1996; Drago 2002). These geographical and climatic features make the island particularly suitable for mosquito introduction and spread. In particular, the main Culicidae species present in Sicily are *Culex pipiens* s.l., *Culex theileri* Theobald 1903*, Ae. albopictus, Culiseta longiareolata* (Macquart 1838), *Culiseta annulata* (Schrank 1776), *Anopheles maculipennis* s.l., *Anopheles algeriensis* Theobald 1903, *Aedes* (*Ochlerotatus*) *caspius* (Pallas 1771)*, Aedes* (*Ochlerotatus*) *detritus* (Haliday 1833). The most relevant mosquito-transmitted disease in the island is the West Nile Fever, caused by the West Nile Virus (WNV), belonging to the *Flavivirus* genus. The virus is maintained by a primary transmission cycle between mosquitoes and wild bird species, acting as amplifying host. Humans, horses and other mammals may be infected by mosquitoes, but they are incidental and dead-end hosts (Di Sabatino *et al.* 2014).

Among mosquitoes, *Ae. albopictus* is the main documented invasive species in the island. Its presence was first detected in Palermo (Sicily) in the autumn 2004, fourteen years after its introduction in mainland Italy (Genoa, September 1990). The species represents an important nuisance as well as a major public health concern due its ability to transmit a large variety of arboviruses (Manica *et al.* 2016).

The first entomological surveillance aimed at assessing *Ae. albopictus* abundance in the island was carried out in 2005 in Palermo municipality, monitoring 28 sites with 300 ovitraps. *Ae. albopictus* was found throughout the city and with a high abundance of eggs (Torina *et al.* 2006). The highest number of eggs per ovitrap was recorded in August and September, suggesting a greatest activity of *Ae. albopictus* in these months, while a significant decrease was observed from November.

Subsequently, during the years 2007 and 2008, an entomological survey was carried out in Agrigento province (South of Sicily) to investigate *Ae. albopictus* abundance and distribution. In

2008, a greater spatial spread of *Ae. albopictus* was observed with more municipalities positive for the presence of this mosquito, not only in proximity of the sea but also in the inland. Indeed, in the first year of survey, 7 out of the 35 monitored municipalities resulted positive for *Ae. albopictus*, while in the second year, 20 out of 39 municipalities were positive. Moreover, even a higher number of eggs per ovitrap were observed in 2008 than 2007. These findings confirmed the high ability of this species to adapt and spread (Cuffaro *et al.* 2009). To date, the species has been found in all the Sicilian provinces, even at medium-high altitudes between 600 and 800 meters above sea level (a.s.l.).

A recent study was carried out to characterise mosquito abundance and microhabitats though a long-time analysis (2009-2019) in an urban restricted context in Palermo municipality (Torina *et al.* unpublished data). A shift of the peak periods for *Ae. albopictus* compared to *Culex* spp. was registered, as *Ae. albopictus* showed its peak between August and October while *Culex* spp. was more abundant in June-July. Moreover, over the years, a progressive decreasing of periods of *Ae. albopictus* absence was observed, probably related to its ability of adaptation to climatic conditions.

Among the minor islands of Sicily, Lampedusa and Linosa (Archipelago of the Pelagie) and Pantelleria are the Italian and European most southern areas, positioned in front of the northern coast of Africa. Lampedusa geologically belongs to the African continent, while Linosa and Pantelleria are of volcanic origin. A recent study, carried out in the summer 2015 detected *Ae. albopictus* on these three islands, indicating new boundaries of the distribution of this species in Europe with Lampedusa representing its southernmost limit (Di Luca *et al.* 2017).

The detection of *Ae. albopictus* even in the Sicilian extreme environmental conditions, such as the ones of the minor islands of Pantelleria, Lampedusa and Linosa, showed that this invasive mosquito was able to arrive and spread even in areas characterised by aridity, strong winds, seasonal lack of water resources, scarcity of green areas and breeding sites. *Ae. albopictus* was mainly found in proximity to human settlements, confirming its ability to progressively adapt to urban and sub-urban habitats (Di Luca *et al.* 2017).

The aggressive and daytime biting behaviour of *Ae. albopictus* in urban habitats represents a significant nuisance capable of modifying human habits, particularly in recreational areas. Moreover, *Ae. albopictus* is a public health concern due to its ability to transmit pathogens. Indeed, it is vector of several arboviruses, such as CHIKV, DENV, and the recently introduced ZIKV. Furthermore, despite *Cx. pipiens* s.l. is the main vector of some zoonotic pathogens, such as WNV and *Dirofilaria* canine nematodes and that this species is highly represented in Sicily, the opportunistic biting behaviour of *Ae. albopictus* could involve this invasive mosquito even in the transmission of these pathogens (Cancrini *et al.* 2003, 2007, Fortuna *et al.* 2015, Manica *et al.* 2016, Richards *et al.* 2006, Valerio *et al.* 2010).

In Sicily, the first outbreak of WNV infection in animals was reported in August 2010, with seven horses showing neurological signs in an area close to Trapani. No human cases occurred in that occasion (Calistri *et al.* 2010). In October 2011, two horses with neurological disorders were observed around Messina and near Palermo, while the first confirmed human case of WNF in Sicily was reported on September 2016 in Trapani (Guercio *et al.* unpublished data).

As concerning filariasis, it is a parasitic disease caused by nematodes of the *Dirofilaria* genus (Spirurida: Onchocercidae), such as *D. immitis* and *Dirofilaria repens* Railliet and Henry 1911.

The disease affects mainly wild dogs and canids. In particular, the foxes may constitute relevant reservoirs for humans and domestic animal parasites since they are widespread in Sicily and they seem to adapt quite well to the human presence, often living in proximity to domestic areas (Torina *et al.* 2013). In Sicily, a large survey was carried out on 2,512 dogs to estimate the prevalence of microfilaremia of different filarial species (Giannetto *et al.* unpublished data). A significantly higher prevalence of *D. repens* (30.8%) in animals was found in Trapani Province compared to other provinces (0.4-4.7%). This patchy distribution of *D. repens* among Sicilian provinces was related to a human case of ocular infection with *D. repens* in Trapani, an area where canine dirofilariasis is endemic (Otranto *et al.* 2011).

A recent study addressing to infer the invasion history and migration patterns of *Ae. albopictus* in Italy confirmed that *Ae. albopictus* expansion has occurred thanks to multiple independent invasions by different colonists from multiple geographic locations. Despite the study reported that the genetic differentiation was related to the geographic distance (northern vs southern cluster regions), Sicilian samples were an exception since they clustered with mosquitoes from northern, rather than southern regions (Pichler *et al.* 2019).

The different ways in which invasive mosquitoes have spread both in Italy and in Europe in recent years have highlighted the extreme variety of entry routes for this species. In Italy, the initial invasion by maritime transportation and local colonisation has been strengthened by passive transportation of mosquito adults by ground moving that played a critical role in the fast-long-range dispersal (Pichler *et al.* 2019).

As concerning Sicily and its minor islands, the maritime route is the most probable pathway of entry (Figure 4), given the high number of containers and goods transported by ship and of direct ferries carrying tourists and their vehicles. The relevant role of ports and airports for the entry of invasive mosquitoes has been also confirmed by a study of the National Centre for Disease Prevention and Control (CCM, Ministero della Salute), carried out in 2014, detecting the presence of *Ae. albopictus* at the port and the airport of Palermo. These findings allow to suppose that also other *Aedes* species with a similar ecology could find a such possible access route. Moreover, *Ae. albopictus* may have reached Linosa from Sicily or Lampedusa, due to the daily connections with these two islands. Regarding Lampedusa, this island represents one of the main entrance points for migrants and refugees into Europe and thus a high-risk area for vector-borne pathogen introduction via migration flows. However, its extreme climatic and environmental conditions as well as the short period of stay of migrants do not favour the transmission of vector-borne pathogens (Di Luca *et al.* 2017).

As regarding the dispersal of *Ae. albopictus* in the different Sicilian provinces, it is probably that an active spreading occurred, as observed in Agrigento province, where not only a spatial dispersal but also an increase of mosquito numbers have been observed.

A national surveillance for WNV in Italy started from 2002 with the National surveillance plan for West Nile type encephalitis (Ministero della Salute 2002). The plan has been progressively improved and extended and today it is integrated in the National Plan of Prevention, Surveillance and Response to Arbovirus 2020-2025. According to this plan, Sicily is considered an area at a high risk of WNV transmission. Therefore, monitoring in this region includes: (1) surveillance of sedentary birds; (2) entomological surveillance; (3) the surveillance of cases of neurological symptoms in equids (only for WNV); (4) surveillance of wild bird specimens found dead; (5) surveillance of cases of human neuro-invasive disease and/or recent infections.

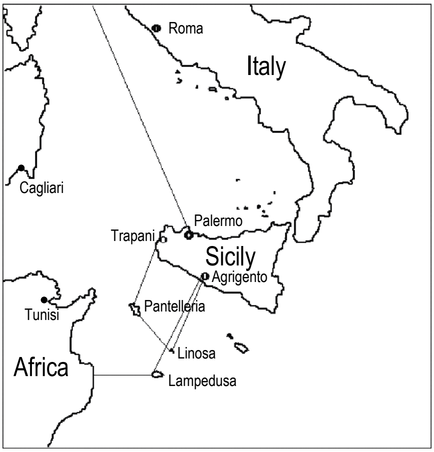

Figure 4. Main maritime trade flows among Sicilian islands that represent probable routes of introduction of invasive mosquitoes.

For the entomological surveillance, the territory is divided into regular areas not exceeding 20 km on each side or 400 km^2 and at an altitude not above 600 m a.s.l. In each identified area, at least one CDC type trap with CO_2, BG-Sentinel or Gravid trap (GAT, Biogents, Germany) is placed. In particular, the Sicilian territory is divided into 15 areas with 2 traps per unit, for a total of 30 active traps in the island. The trap must be active consecutively for 24 hours and the catches are carried out every two weeks at least from April to November.

European (EU Regulation No. 1143/2014) and Italian (Legislative Decree 15 December 2017, 230) legislations highlight the significant negative effects on human health, economy and biodiversity caused by the introduction of invasive species and they aim to prevent and mitigate these effects. In particular, the Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) is the Italian technical body in charge of providing scientific support and of establishing a surveillance system for invasive alien species. The network of Experimental Zooprophylactic Institutes provides the Italian Regions with the technical-scientific contribution for entomological surveillance activities.

In 2018, a National plan for surveillance and response to arbovirosis transmitted by invasive mosquitoes, with particular reference to CHIKV, DENV and ZIKV, was established by the Italian Ministry of Health (Ministero della Salute 2018, 2019).

Volcanic islands

Main and potential culicids vectors of zoonosis in Cabo Verde

Cabo Verde is a small island country formed by ten islands, located 560 km west of the Senegal coast and made up of volcanic origin islands (Madeira 2002). The country is mountainous and furrowed by erosion and volcanic activity. The climate is characterised as semi-arid, with annual averages of 25 °C (temperature) and 250 mm (rainfall). Local climatic conditions vary between islands.

It is estimated that the culicids arrived in Cabo Verde along with the first human populations in 1460, but the first records date from the 20th century. Between 1909 to 1964, the Lisbon Institute of Hygiene and Tropical Medicine (IHMT) registered the presence of six species of culicids. In 1977, the number increased to eight (Ribeiro *et al.* 1980). Since then, except for the identification of *Culex* (*Lutzia*) *tigripes* (De Grandpre and de Charmoy 1901) as a new species introduced on the island of Maio (Cambournac *et al.* 1984), there have been no records of other national studies until 2007. This was carried out by the Ministry of Health, with an identification of a new species (Alves *et al.* 2010). The last update of the culicidological fauna was carried out in 2017 by the National Institute of Public Health (Leal *et al.* 2021) (Table 1).

Anopheles arabiensis was the first mosquito registered in Cabo Verde, in 1909, on the island of Santiago, originating from Senegal (Cambournac *et al.* 1982). It has spread to almost the entire archipelago (Table 1), being the main and only known vector of malaria in the country. The presence of *An. arabiensis* has been recorded in all the islands, except Sal and Brava (WHO 2012). It was also a vector of lymphatic filariasis, currently eradicated (Franco and Menezes 1955). *An. arabiensis* could be involved in the transmission of dirofilarial infections in dog populations on the island of Maio (Marcos *et al.* 2017), requiring vector competence studies to confirm this hypothesis. In Cabo Verde, it has an exophilic, endo-exophagic behaviour and a reduced anthropophilic bite preference with a predominance of mixed blood meals (Dias 2020, INSP 2018, Ribeiro *et al.* 1980). Regarding the type of habitat, the larvae are found in different larval habitats: irrigation waters, wells and water tanks, temporary larval habitats such as puddles and leaves on the ground (Correia *et al.* 2015, Ribeiro *et al.* 1980). Populations of *An. arabiensis* are affected by seasonality,

Table 1. Distribution of the culicidological fauna of Cabo Verde. Brava (B), Fogo (F), Maio (M), Santiago (S), Boa vista (BV), San Vicente (SV), Santo Antão, (SA), São Nicolau (SN).

Species/subspecies	Distribution 1909-1964	Distribution 1965-1977	Distribution 1978-2007	Distribution 2007-2018
Anopheles arabiensis (Patton 1905)	B, M, S, Sal, BV, SV	B, M, S, Sal, BV, SV	SN, BV, M, S, Sal, F, B	S, M, SV, BV, F, SN, SA
Anopheles pretoriensis (Theobald 1903)	SN, SV	SN, SV, M, F, B	SA, BV, M, SV, SN, F, B, S	All the islands
Aedes caspius meirai[1] ssp.	Sal	Sal, BV, M.	BV, M, Sal, S, F	S, F, M, Sal, BV, SV
Aedes aegypti	B, F, S, Sal, BV, SN, SV, SA	B, F, S, Sal, BV, SN, SV, SA	SA, SN, M, S. F, B	All the islands
Lutzia tigripes (de Grandpre and de Charmoy 1901)			M, S, Sal, F	S, BV, SV, SN, SA
Culex perexiguus (Theobald 2003)			S	S
Culex tritaenorhynchus (Giles 1901)				S
Culex bitaenorhynchus (Giles 1901)		SA, S	SA, S	SA, S
Culex pipiens s.s. (Linnaeus, 1758)		BV, SA, M, S	All the islands	All the island
Culex quinquefasciatus (Say 1823)	SN, Sal, B	SN, SA, Sal, F, B		
Culiseta longiaerolata	SN, SV, Sal	SA, SV, S N, M, Sal, S, F, B	SA, SV, SN, BV, Sal, M, F, B	SA, SN, Sal, S, B

[1] Endemic subspecies of Cabo Verde.

especially in the driest islands and localities. Due to the profile of autochthonous malaria cases, it is estimated that it has nocturnal biting habits.

Aedes aegypti was registered by Sant'Anna in Cabo Verde (1930) and is the main vector in the country of YF in the past and dengue and Zika epidemics in 2009 and 2015 respectively (Franco *et al.* 2010, MSSS 2016, Patterson 1988, Ribeiro *et al.* 1980). It is considered a competent vector of CHIKV (Diagne *et al.* 2014). Regarding its origin, it was probably introduced from the coast of West Africa, considering the possibility of several reintroductions (Campos *et al.* 2020, Patterson 1988). This species is found in the entire archipelago, although with differences between locations and time periods (Table 1). In the last survey the islands of Brava and Santiago presented the highest number of breeding sites (Leal *et al.* 2021). It is considered anthropophilic, domestic, occurring mainly in artificial, domiciliary or peri-domiciliary breeding sites (Correia *et al.* 2015, Dias 2020, Gómez *et al.* 2015, Ribeiro *et al.* 1980). They prefer fresh, clean waters, with low concentration of salts and neutral PH (Ribeiro *et al.* 1980). However, in some locations they have been observed in larval habitats with organic matter and dissolved solids (Correia *et al.* 2015, INSP 2018, Leal *et al.* 2021).

In Cabo Verde, vector control began in 1948 with the malaria eradication campaigns, until 2009 only targeting *An. arabiensis*. This consisted of intradomiciliary spraying (PID) with DDT, larval habitats treatment with petroleum derivatives and use of *Gambusia affinis* in agricultural areas (MSSS 2018). In 1999, Deltamethrin replaced DDT and the larvicide Temephos was introduced in 1979 (Cambournac *et al.* 1984). In addition, is performed spatial spraying with Deltamethrin during outbreaks, health education and social mobilisation. Vector control is part of the Integrated Program Against Vectors Diseases of the Ministry of Health. The activities are financed mainly by the Government, with the help of national and international institutions. Entomological surveillance has improved in recent years with the creation, in 2017, of the Laboratory of Medical Entomology of the National Institute of Public Health, although this is still incipient, without a continuous vector monitoring system established for the entire national territory.

Published studies on the resistance of culicid vectors to insecticides in Cabo Verde date back to 2009, with the first dengue epidemic. For *Ae. aegypti*, resistance to the adulticides DDT, propoxur, deltamethrin and cypermethrin have been described (Dia *et al.* 2012, Rocha *et al.* 2015). Metabolic detoxification systems, such as glutathione-S-transferase (GST) and mixed function oxidases (MFO) enzymes appear to be involved (Rocha *et al.* 2015). Low frequency *kdr* mutations have also been observed recently (Ayres *et al.* 2020). For *An. arabiensis*, resistance to deltamethrin 0.05% and to malathion 5% has been recorded, with *kdr* mutations involved in this phenotype (Da Cruz *et al.* 2019, De Pina *et al.* 2018). In relation to larvicides, different studies showed resistance of *Ae. aegypti* and *An. arabiensis* to temephos (Pires *et al.* 2020, Rocha *et al.* 2015).

The changes in temperature and rainfall projected for the country, which are already being verified, may favour a greater abundance and dissemination, both of vectors and pathogens. A reduction in the water capacity implies an increase in the storage of water by the population, which is one of the anthropic factors that favour vector-borne diseases in Cabo Verde. In turn, the IPCC (Intergovernmental Panel on Climate Change) projections for the Sahel point to a rainfall reduction of 10-20% in 2050 and an increase in the country's average temperature from 25.1 to 28 °C (Niang *et al.* 2014). This rise will favour an increase in vector and waterborne diseases, such as malaria, dengue, yellow fever, diarrheal diseases, etc.

Current situation in the Canary Islands regarding the monitoring of invasive mosquito species

The emergence of yellow fever epidemics in the Canary Islands was especially noteworthy between 1810-1811 and 1862-1863 in the city of Santa Cruz de Tenerife, with 1,450 and 540 deaths respectively, an important proportion of the population at that time (11,000 inhabitants). The main cause of these outbreaks was related to the intense maritime connection between the islands and Central and South America. The last references to the existence of cases of this disease date back to the last century (Fernández-López and Sierra-López 1973), being the vector the species *Ae. aegypti*. However, as a result of the actions taken in the fight against malaria in the 1950s, *Ae. aegypti* was eradicated between 1970 and 1971. At that time, the World Health Organization (WHO) declared the international airports of Santa Cruz de Tenerife and Las Palmas de Gran Canaria free of *Ae. aegypti* (WHO 1972). Furthermore, aircrafts passing through these airports were therefore exempted from the application of Articles 74.4 and 96.2 of the International Health Regulations.

Currently, the Canary Islands continue to be free from the presence of invasive mosquito species. Since 2013, and following the dengue outbreak in 2012 in the archipelago of Madeira, with 1,080 confirmed cases of serotype 1 (DENV-1) (Auerswald *et al.* 2019), the Canary Islands set up an Entomological Surveillance system in Ports and Airports to control the introduction of alien mosquito species that transmit pathogens. This surveillance is part of the Entomological Surveillance in Ports and Airports for Imported Vectors of Exotic Infectious Diseases, and Surveillance for Potential Autochthonous Vectors of these Diseases (Lucientes and Molina 2017).

During the first year of the Surveillance Network, the entry points at the islands of Tenerife, Gran Canaria and La Palma were monitored since they represent the highest air and maritime traffic in the area. During the following years and until 2016, the islands of Lanzarote and Fuerteventura were incorporated into the Surveillance Network.

Up to date, 17 Points of Entry (PoE) are monitored in the archipelago (Table 2) with a periodicity of between 10-15 days depending on the climatic characteristics of the area. The samples are mostly collected at the ports and airports, as well as greenhouses. The introduction of invasive species could be due to the transportation of ornamental plants, as in the case of *Ae. albopictus* detected in Holland in 2005 in imported lucky bamboo (*Dracaena sanderiana*) (Scholte and Schaffner 2008).

The Surveillance Network is developed throughout the year in all the PoE due to the optimal climatic conditions of the Canary Islands, which favour presence of mosquitoes in all seasons. In addition to the surveillance for invasive species, native species of mosquitoes, such as *Cx. pipiens* s.l., are also surveyed to assess its role as vector of arboviruses WNV (MHCSW 2014).

Also noteworthy is the presence in the archipelago of species which could be potential vectors of malaria, such as *Anopheles cinereus hispaniola* (Theobald 1903) and *Anopheles sergentii* (Theobald 1907) (Baez 1987, Baez and Fernández 1980, Eritja *et al.* 2002), whose populations are regularly monitored as part of the surveillance of native species.

The main trapping methods used in the Canary Islands for the surveillance of invasive species following the guidelines of the European Centre for Disease Prevention and Control (ECDC 2012) and considering absence of invasive vectors (Scenario 1). The collected samples are sent to the Medical Entomology Laboratory of Canary Islands University Institute of Tropical Diseases and Public Health located at the University of La Laguna (IUETSPC) in the island of Tenerife.

Table 2. List of points of entry (PoE) monitored on each island.

Island	Points of Entry (PoE)
Fuerteventura	Airport of Fuerteventura Port of Puerto del Rosario Greenhouse 'Fuerteventura Garden' Urbanisation Las Granadas (Puerto del Rosario)
Gran Canaria	Airport of Gran Canaria Port of Las Palmas Greenhouse 'El Rosal'
Lanzarote	Airport of Lanzarote Port of Arrecife Greenhouse 'Las Palmeras' (Arrecife)
La Palma	Port of Santa Cruz de La Palma Greenhouse 'Albelfra S.L.'
Tenerife	Tenerife North Airport Tenerife South Airport Port of Santa Cruz de Tenerife Greenhouse 'Dragoflor S.L.' Greenhouse CATESA

Mosquito adults are identified morphologically (Becker *et al.* 2010) or by molecular techniques for cryptic species or damaged specimens. The results are published annually in the Entomological Surveillance report of the MHCSW.

Thanks to the Entomological Surveillance, the invasive species *Ae. aegypti* was detected in the island of Fuerteventura in December 2017. Due to the prompt action and involvement of the competent authorities, the immediate delimitation and control using adulticides and larvicides of the population restricted to a small urbanisation of the municipality of Puerto del Rosario was possible. The captured specimens were sent to IUETSPC where molecular techniques confirmed the morphological identification and infection by *Alphavirus* or *Flavivirus* was discarded. Molecular analyses also detected the presence of resistance mutations to insecticides, such as DDT or pyrethroids. Surveillance on the island was intensified both in the number of traps and in the frequency of sampling in order to be able to control their possible dispersion to other municipalities and even to other islands. In June 2019, the eradication of *Ae. aegypti* from the archipelago was declared after 18 months without positive findings by the Surveillance Network.

Currently, the Canary Islands do not have a Plan for Preparedness and Response to Vector-Borne Diseases of the *Aedes* genus. However, it will be prepared thanks to the recent funding from the European Regional Development Fund (ERDF) within the Territorial Cooperation Program INTERREGVA Spain-Portugal MAC 2014-2020 for the achievement of the project called Institutional Cooperation to increase the effectiveness and efficiency of the Public Health policy linked to the introduction of Tropical Diseases in Macaronesia (AEDENET).

Aedes aegypti in Madeira Island

Madeira is the main island of the Autonomous Region of Madeira. This region, constituted by two inhabited islands (Madeira and Porto Santo) and two groups of smaller islands or islets (Desertas and Selvagens) is located in the Atlantic Ocean, ca. 685 km from the West coast of Africa. The archipelago of Madeira is part of the Macaronesia region together with the archipelagos of Canarias and Cape Verde. Human population totalises 254,254 residents with 104,024 inhabitants living in the capital city, Funchal (DREM 2019). Madeira Island has typical volcanic structure with a hilly inner region and two main coasts; the Northern one cooler and rainier than the Southern coast. Madeira is considered to have a very mild and moderated subtropical climate, with dry Mediterranean summers and rainy winters. According to Koppen classification, Madeira has a Csa, temperate climate (IPMA 2021), however, microclimates are common at any altitude.

The archipelago presents a relatively low number of culicid species, most of them neither particularly aggressive nor abundant to be considered a nuisance species. Thus, complaints regarding an aggressive mosquito that was causing severe allergic reactions due to its bites, let to an entomological survey based on which the presence of *Ae. aegypti* was first recorded in the archipelago of Madeira (Margarita *et al.* 2006). In 2006, the species was detected in several parishes of Funchal, being particularly abundant in the area of St. Luzia. However, reports from the community and medical records indicate that the introduction of *Ae. aegypti* may have occurred in 2004 (Almeida *et al.* 2007). Control measures were rapidly implemented by local health authorities, 'Instituto de Administração da Saúde (IASAUDE, IP-RAM)' in order to contain or even eradicate the invasive species. The intensive vector control program was based in two main pillars: (1) health education campaigns to engage the local community in source reduction activities and to promote the use of personal protection measures; (2) insecticide-based strategies with the use of chemical adulticides in indoor and outdoor sprayings (pyrethroids and organophospates) and biological larvicides as *Bti* (ECDC 2014). A vector surveillance program based on a network of ovitraps was also implemented in collaboration with Natural History Museum of Funchal. Currently, this surveillance program is composed by 2 islands-wide (Madeira and Porto Santo) networks of 191 ovitraps and 24 BG-traps placed in Funchal area and points of entrance, as harbours and the International Airport (IASAUDE, 2020). Ovitraps are made of a 10l black bucket, with a 50 cm ruler covered by 3 cm-width stripe of red velvet paper. The adult BG-traps are baited with lure but without CO_2. Regardless all control efforts, *Ae. aegypti* expanded to the West and East of Funchal city and, in 2008, was recorded in the neighbouring counties of Câmara dos Lobos (West) and Santa Cruz (East) (Gonçalves *et al.* 2008). Community-based strategies became the major control tool when, in 2009, WHO bioassays (IASAUDE 2020, WHO 1998; 2016) revealed the presence of insecticide resistance to pyrethroids and DDT in *Ae. aegypti* population of Funchal (Seixas 2012). The distribution pattern of the species was updated in 2011, during an-island wide survey (Nazareth *et al.* 2014). Results showed that *Ae. aegypti* had spread along the Southern coast of the island been present from the county of Calheta, (West), until Machico (East). In 2020, the species distribution pattern, with small yearly variations is similar to what was observed in 2011-12 (ECDC 2014, IASAUDE 2020, Nazareth *et al.* 2014). The species was detected in three different occasions in the north coast of the island but, apart these sporadic introductions, the species was never able to colonise the Northern and inland regions of the island (IASAUDE 2020) possibly due to the cooler climate and to the altitude effect since no specimen was ever collected above 200 m (IASAUDE 2020).

Aedes aegypti originally from West African species has spread to several regions of the Globe since the 15-17th centuries. Although relatively close to the West coast of Africa, the first genetic studies of the introduced population in Madeira suggested South America, namely Brazil and Venezuela, as the most likely source (Seixas *et al.* 2013). This conclusion was based on mtDNA studies, *kdr* mutations associated with insecticide resistance and socioeconomic information that has revealed a high level of human flux and goods trade between Madeira Island and the two mentioned countries. Further studies where carried-out involving 16 microsatellites and two mitochondrial DNA genes, analysed in samples of Madeira Island from different years (2005, 2009, 2011, 2012, 2013 and 2014) and from three different locations: Paul do Mar, located at the most Western region of *Ae. aegypti* distribution area; Funchal and the neighbouring county of Santa Cruz, where the International Airport is located. Results showed the occurrence of, at least, two independent colonisation events, both from a South American source population(s), most probably from Venezuela (Seixas *et al.* 2019).

Like in other regions of the world, *Ae. aegypti* breeds mainly in domestic and peridomestic containers. Being an island with high abundance of fresh water and piped water systems in all counties, there is not the generalised habit of collecting water in drums or plastic containers, at least in the urban areas where *Ae. aegypti* is most abundant. Therefore, the main larval habitats are small containers associated to gardens or verandas, namely the dishes of flowerpots, which are common in almost all households. In public areas, one of the most common breeding places is storm drains. Studies carried-out in 2012 showed that 15.7% of storm-drains of downtown Funchal were colonised with mosquito larvae, 12.2% of them with immatures of *Ae. aegypti* (unpublished data). Public fountains and small lakes have running water or are colonised with indigenous predator fishes and, thus, not considered productive larval habitats.

Aedes aegypti is generally considered an endophagic and endophlic species. Regarding its biting habits in Madeira, this species presents a diurnal pattern with peaks of activity after dawn and during the sunset (unpublished data). However, biting activity can linger during several hours after nightfall. Although being known by its endophagic behaviour, Madeira´s population can easily feed both indoors and outdoors (unpublished data). When comparing biting patterns estimated in indoor and outdoor collections, light differences can be observed. Although indoors human-biting-rates are higher than outdoors estimates they are not statistical different (unpublished data). Indoors biting pattern present a late morning (11:00-12:00 h) and a precocious evening peak (17:00-18:00 h) when compared with outdoors biting activity (8:00-9:00 h and 18:00-19:00 h).

Based on long-established surveillance activities, seasonal activity of *Ae. aegypti* in Madeira is well defined. The population presents higher abundance levels between September and November and lower densities in the months of February and March. Seasonality presents small yearly differences probably due to the influence of meteorological factors as rainfall and temperature (ECDC 2014; IASAUDE 2020).

In 2012, the first and only outbreak of an *aegypti*-borne disease was recorded in the archipelago (Sousa *et al.* 2012). That year was characterised by atypical summer rainfalls and higher mean temperatures in the months prior to the outbreak (ECDC 2014). In August, vector densities were already abnormally high and control activities were reinforced. Despite the health authorities' efforts, on the 3rd of October a dengue outbreak was officially declared with its highest incidence rates located in Santa Luzia and Nazaré neighbourhoods, Funchal city. This outbreak, which ended in March of 2013, totalised 1,080 confirmed cases, the majority of them recorded in Funchal (77%) (ECDC 2014). However, a serological survey carried-out in 2016, has assessed the real impact by estimating a 7:1 ratio of subclinical to symptomatic infection outcomes (Auerswald *et al.* 2019). Dengue virus 1, with phylogenetic relationships with South America, was identified as the etiological agent (Alves *et al.* 2013) and *Ae. aegypti* was the confirmed vector through virus detection in several groups of specimens (unpublished data). Due to touristic activities, 81 imported cases with origin in Madeira Island were recorded in 14 different countries of Europe (Auerswald *et al.* 2019).

With the presumed competence for DENV-1 transmission already established, two populations of *Ae. aegypti* from Madeira were tested to evaluate their vector competence regarding other arboviruses. Results showed that both populations could transmit ZIKV, CHIKV and DENV-2 viruses, although with different vector competence according to the population and virus tested (Jupille *et al.* 2016, Seixas *et al.* 2018).

Following the preliminary studies carried out in 2009, several insecticide susceptibility bioassays were carried-out along the years, testing different populations and compounds. In general, Madeiras´s populations are resistance to insecticides of all classes used in Public Health, including permethrin, cyfluthrin, deltamethrin, fenitrothion, bendiocarb and DDT. Both metabolic and target-site resistance mechanisms were detected. Preliminary studies showed significantly higher mortality rates in synergists tests and elevated activities of detoxification enzymes in biochemical bioassays, strongly suggesting the presence of metabolic resistance (Seixas *et al.* 2017). Further studies of microarrays-based transcriptome have confirmed these preliminary results. Significant upregulation was found in 11 genes associated to the 3 detoxification enzyme super-families (P450s, GSTs and esterases), as well as in eight transcripts encoding putative cuticle proteins and two others encoding hexamerins, thus confirming the existence of several metabolic mechanism associated with insecticide resistance. As to target-site resistance, following the preliminary results of 2013 studies (Seixas *et al.* 2013), genotyping of *kdr* loci revealed the presence of a fixed mutation, F1534C, and the existence of V1016I mutation which frequency has increase over the years (Seixas *et al.* 2017). Subsequent studies have also revealed the presence of a third recently described *kdr* mutation, V410L, in similar, moderated, frequencies of V1016I (Ayres *et al.* 2020).

Insecticide resistance imposes serious restrictions to vector control and neutralises the major tool to be used during epidemics, rendering prevention a major role in the control of *Ae. aegypt*i-transmitted diseases. Thus, in Madeira the ongoing arboviruses control program relies mainly in community-based vector control strategies and in well-established entomological and epidemiological surveillance systems, both incorporated into a multi-sectorial strategy of monitoring, early detection and control.

Invasive species of Hawaii, USA: a case study on the introduction of *Aedes japonicus*

The Hawaiian Islands are located in the Pacific Ocean about 4,000 km from California on the mainland. The state of Hawaii is made up of eight major islands: Hawaii (Big Island), Maui, Kauai, Oahu, Molokai, Lanai, Niihau and Kahoolawe. The ~28,000 km^2 of islands are home to approximately 1.4 million people (U.S. Census Bureau 2020). However, Hawaii is not home to any native species of mosquito. In fact, the islands were free of mosquitoes until the first half of the 19th century (Hardy 1960). The tropical Hawaiian climate has moderate temperatures that fluctuate marginally throughout the year. While summer has the most ideal temperatures for mosquito development, the winter is characterised by significant rainfall. The combination of these characteristics allows for continual development of mosquitoes throughout the year (Leong and Grace 2009, Liao *et al.* 2015).

There are six invasive species of mosquitoes on the Hawaiian Islands and one genus of mosquito that was intentionally introduced (Winchester and Kapan 2013). The first invasive mosquito to be detected in the islands was *Cx. quinquefasciatus* in 1826, which made its entry via water containers on a ship from Mexico (Hardy 1960). The next unintentional introduction was not observed until the 1890s when *Ae. aegypti*, arrived and was soon followed by *Ae. albopictus* in the 1900s (Hardy 1960, Winchester and Kapan 2013). The next introduction, *Aedes vexans nocturnus* (Theobald 1903), an inland floodwater species, came to Oahu approximately 60 years later (Joyce and Nakagawa 1963) and in 1981, *Wyeomyia mitchellii* (Theobald 1905), a bromeliad-inhabiting mosquito, was discovered on the same island (Shroyer 1981). The most recent detection of an invasive mosquito species in Hawaii was *Ae. japonicus japonicus* (Theobald 1901), the rock-pool mosquito or Asian bush mosquito, which was first detected in 2003, and has since expanded its range (Larish and Savage 2005, Larish *et al.* 2010). It is unclear exactly how this species made its way into the Islands.

In addition to these six established and invasive species, there have been sporadic detections of *Anopheles* mosquitoes in Hawaii since 1929 (Van Zwaluwenburg 1929), with the most recent documented detection occurring in 2003 (Furumizo *et al.* 2005). There are currently no established populations of *Anopheles* in Hawaii (Winchester and Kapan 2013). Additionally, since 1929, four species in the genus *Toxorhynchites* (Theobald 1901), have been intentionally introduced to the islands (Winchester and Kapan 2013). *Toxorhynchites* are predacious on other mosquitoes as larvae and were introduced to aid in controlling the pestiferous mosquitoes on the islands and based on surveillance information, at least two *Toxorhynchites* species are still common in Hawaii (Winchester and Kapan 2013).

Except for *Wy. mitchellii*, all the invasive mosquitoes of Hawaii are significant vectors of human and/or animal pathogens. Since their introduction, *Ae. aegypti* and *Ae. albopictus* have been responsible for transmitting DENV to humans (Morris 1995, Winchester and Kapan 2013). The first major epidemic of dengue in Hawaii resulted in 30,000 cases (Wilson 1904) and most recently, mosquito-borne transmission of dengue occurred in 2016 (State of Hawaii Department of Health 2019). In addition to human pathogens, there is endemic transmission of *D. immitis,* the causative agent of dog heartworm. *Cx. quinquefasciatus*, *Ae. aegypti*, *Ae. albopictus* and *Ae. vexans* (Meigen 1830) are all considered vectors of *D. immitis* (Ledesma and Harrington 2011) and *Ae. japonicus* may also be a significant vector (Silaghi *et al.* 2017). Perhaps the most significant mosquito-borne disease transmitted to animals in Hawaii is avian malaria. Avian malaria is caused by *Plasmodium* parasites such as *Plasmodium relictum* and is particularly detrimental to passerine birds that have not co-evolved with the parasite (LaPointe *et al.* 2012). The introduction of avian malaria transmitted primarily by *Cx. quinquefasciatus*, to Hawaii has been implicated in the decline and extinction of several native bird species (LaPointe *et al.* 2012, Samuel *et al.* 2011, Warner 1968).

Of the invasive species in Hawaii, *Ae. japonicus* presents the greatest level of uncertainty when it comes to its role as an arbovirus vector in the state. Laboratory studies have shown *Ae. japonicus* to be competent vectors of WNV, La Crosse virus, eastern equine encephalitis virus, St. Louis encephalitis virus, DENV and CHIKV (Schaffner *et al.* 2011) and could be a significant vector of Japanese encephalitis virus in the absence of the primary vector (summarised in Larish and Savage 2005).

Ae. japonicus eggs are desiccation resistant and larvae have been collected from infusion-baited water containers, surface depressions, potholes, tractor tires, plastic tarps and an abandoned fishpond in the Hawaiian Islands (Harwood *et al.* 2018, Larish and Savage 2005). Adults have been collected from New Jersey Light Traps (NJLT) and infusion-baited gravid traps (Harwood *et al.* 2018, Larish and Savage, 2005, Larish *et al.* 2010). Primary blood hosts are mammals (mostly human), but they will also feed on birds (summarised in Kaufman and Fonseca 2014). The adult females are distinguished from the other three *Aedes* species that occur in the islands by the presence of golden-coloured scales that form a lyre-shaped pattern on the scutum; this pattern is formed by silver scales in *Ae. aegypti* and is not present in *Ae. albopictus* or *Ae. vexans*.

In Hawaii, *Ae. japonicus* was first detected in a NJLT by the Vector Control Staff of the Hawaii Department of Health in November of 2003 (Larish and Savage 2005). Surveillance conducted around the Island of Hawaii in 2004 yielded collections of *Ae. japonicus* from several sites using both NJLTs and gravid traps (Larish and Savage 2005). Since those initial detections, additional surveillance has been conducted on two other Hawaiian Islands, Oahu and Kauai (Harwood *et al.* 2018). During this surveillance, artificial larval habitats, CDC light traps baited with a BG lure and dry ice, BG-Sentinel traps baited with a BG lure, gravid traps baited with timothy grass water infusion and larval surveillance were utilised. *Ae. japonicus* was detected on Oahu and Kauai, but only in certain traps. CDC light traps and BG-Sentinel traps did not capture *Ae. japonicus* even when placed in areas that were near larval habitats (Harwood *et al.* 2018). This observation is also reflected in Kaufman and Fonseca (2014) review that summarised the difficulty of collecting adult *Ae. japonicus* using traditional surveillance methods. While larval surveillance seems to be the most sensitive to detecting the presence of *Ae. japonicus*, gravid traps baited with infusions (using leaf detritus, grass, etc.) are the most efficient method of collecting adult *Ae. japonicus* (Harwood *et al.* 2018, Kaufman and Fonseca 2014, Larish *et al.* 2010).

The history of organised mosquito control in Hawaii has been dynamic, at times nearly non-existent and at other times, well-funded and characterised by access to extensive control resources. A more detailed history of mosquito control in Hawaii can be found in Winchester and Kapan (2013). Briefly, the driving force behind much of the mosquito control activities in Hawaii have been, and continue to be, driven by public health threats. Through the years, significant resources were dedicated to training and implementing control and eradication programs to prevent or cease the transmission of pathogens such as DENV (Winchester and Kapan 2013). However, due to budget cuts after the 2008 recession, the State eliminated many positions within the Hawaii Department of Health (HIDOH) Vector Control Program but didn't fully eliminate it (Winchester and Kapan 2013). Following this, a multi-agency partnership between the remaining HIDOH Vector Control Program was formed with the Navy Environmental Preventative Medicine Unit Six, and the United States Geological Survey, which together, were able to conduct thorough surveillance throughout Hawaii (Harwood *et al.* 2018). At the time this chapter was written (2020), the HIDOH Vector Control Program had been fully restored to the pre-recession staffing. Surveillance remains an important component and the program actively participates in improving biosecurity through their mosquito surveillance activities (Hawaii Invasive Species Council 2020). In addition to their rigorous surveillance, the Vector Control Program also takes a proactive role in source reduction, larviciding, and public outreach (State of Hawaii Department of Health 2020a, 2020b). Chemical treatments targeting adult mosquitoes are primarily backpack treatments using residual insecticides, known as barrier treatments (Khon2 News 2015). More emphasis is placed on these chemical control methods during times of local transmission of mosquito-borne diseases. In the recent transmission of DENV, these treatments included a focus on the residential communities where transmission was occurring and targeting the container mosquito vectors in the area. Much of the control of *Ae. japonicus* focuses on source reduction. Interestingly, conservationists in Hawaii have also taken an interest in mosquito control. The transmission of avian malaria to the native birds of Hawaii has led to a movement by conservationists to return Hawaii to a mosquito-free state (Revive and Restore 2017).

Prior to the establishment of the multi-agency partnership in Hawaii, state-wide mosquito surveillance was problematic due to the lack of resources. In addition, the difficulty in detecting *Ae. japonicus* in traditional surveillance traps made early detection of this species difficult (Harwood *et al.* 2018, Kaufman and Fonseca 2014). Early detection of *Ae. japonicus* in Hawaii and swift control actions would have made eradication efforts more feasible (Unlu and Farajollahi 2012), but now that it is established in Hawaii, its cryptic nature makes control even more challenging. However, Chapter 321-11 of the Hawaii Revised Statutes gives the authority to the Hawaii Department of Health to manage 'mosquito breeding habitat' as well as 'deinsectisation of aircraft' to prevent the introduction of vectors or the pathogens that they transmit (Hawaii Revised Statutes 2019). Additionally, significant effort is dedicated to surveillance, source reduction, and regular larviciding at ports of entry. In this way, the Hawaii Department of Health could respond to new introductions of invasive species as well as manage existing populations of mosquitoes on the Hawaiian Islands.

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Zoonotic diseases: new tools for mosquito control

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11. The use of artificial intelligence and automatic remote monitoring for mosquito surveillance

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Abstract

Mosquito surveillance consists in the routine monitoring of mosquito populations: to determine the presence/absence of certain mosquito species; to identify changes in the abundance and/or composition of mosquito populations; to detect the presence of invasive species; to screen for mosquito-borne pathogens; and, finally, to evaluate the effectiveness of control measures. This kind of surveillance is typically performed by means of traps, which are regularly collected and manually inspected by expert entomologists for the taxonomical identification of the samples. The main problems with traditional surveillance systems are the cost in terms of time and human resources and the lag that is created between the time the trap is placed and collected. This lag can be crucial for the accurate time monitoring of mosquito population dynamics in the field, which is determinant for the precise design and implementation of risk assessment programs. New perspectives in this field include the use of smart traps and remote monitoring systems, which generate data completely interoperable and thus available for the automatic running of prediction models; the performance of risk assessments; the issuing of warnings; and the undertaking of historical analyses of infested areas. In this way, entomological surveillance could be done automatically with unprecedented accuracy and responsiveness, overcoming the problem of manual inspection labour costs. As a result, disease vector species could be detected earlier and with greater precision, enabling an improved control of outbreaks and a greater protection from diseases, thereby saving lives and millions of Euros in health costs.

Keywords: mosquito monitoring, remote surveillance, acoustic sensor, optical sensor, intelligent sensor, smart trap, machine learning, Internet of Things (IoT)

Mosquito surveillance and traditional monitoring methods

Mosquitoes (*Diptera, Culicidae*) are responsible for the transmission of diverse medically and veterinary important disease agents (viruses, protozoans and other parasites) which cause serious diseases in humans and animals, such as malaria, dengue, Zika, yellow fever, chikungunya, West Nile virus, Eastern equine encephalitis or avian malaria. Entomological surveillance plays a key role in human and veterinary disease surveillance within the framework of the 'One Health' concept, where interdisciplinary collaboration and communication in healthcare is crucial (ECDC 2012, 2014, WHO 2017). A paradigmatic example of this 'One Health' approach would be the West Nile Virus (WNV) surveillance. This implies a coordinated strategy of Public Health actors that carry out

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the diagnoses of possible infected horses and humans that are dead end hosts of the pathogen; the monitoring of *Culex* mosquitoes that may act as vectors in the areas with WNV cases; and the detection of possible infected birds which may act as reservoirs of the virus.

Mosquito surveillance methods should provide clear and meaningful information for program managers and policy-makers for the purpose of: (1) determining and quantifying the composition of mosquito populations which are present in a specific area; (2) monitoring changes in mosquito populations; (3) identifying the presence of new invasive mosquito species which can act as disease vectors; (4) detecting mosquito-borne diseases; (5) determining which control measures need to be conducted; (6) performing the quality assessment of control measures; and (7) designing accurate risk assessment programs in order to prevent and manage potential disease outbreaks (Flores 2015, Schaffner *et al.* 2014).

Mosquito surveillance can be understood as a task involving the routine monitoring of immature stages and adult mosquito populations over the course of an entire mosquito season (Flores 2015, Silver 2008). Several methodologies have been developed to sample and analyse different stages of the biological cycle of mosquitoes (egg, larvae and adults), although most of them mainly target adults since only adult female mosquitoes are responsible for disease transmission (Focks 2003, Sivagnaname and Gunasekaran 2012). Thus, with the exception of pathogen monitoring in immature stages to investigate vertical transmission, adult mosquito surveillance is probably the most precise approach to properly monitor mosquito populations for vector-borne disease (VBD) risk assessment. While some methodologies focus on resting mosquitoes, such as aspiration in vegetation that is performed with entomological aspirators, most have been developed to catch flying mosquito females when seeking hosts for blood feeding or gravid females when seeking oviposition sites (Becker *et al.* 2010, Service 1993).

To allow standardised monitoring of adult mosquito populations, many types of traps have been developed to attract different target species. Some rely solely on a conventional incandescent filament light bulb as the main source of attraction or use an ultra-violet light source while others add CO2 or chemical attractants to the light source. Various models are commercially available. The most popular are adapted models of CDC mosquito light-traps, EVS trap (Figure 1) and in the last decade, BG sentinel traps (Figure 2) with different combinations of light, CO_2 and chemical lure (EMCA 2020). Other traps include the Reiter trap for gravid females, and even types developed not just for mosquito sampling but for mosquito control as well, such as the Mosquito Magnet™, among others. Despite these methodologies, during the first decade of the 21st century the need became evident for a much greater effort to develop, manufacture and market new tools that would be effective for different species and environmental conditions and that could be standardised in different countries in order to obtain more significant and comparable data (Qiu *et al.* 2007).

Several studies have compared the efficacy of different commercial trapping devices, reporting differences in both performance and efficacy depending on the target mosquito species, the type of attractant and other environmental factors (Brown *et al.* 2008, Li *et al.* 2016, Lühken *et al.* 2014, Roiz *et al.* 2012). Generally, BG-traps have shown better, or at least a similar performance, compared to CDC, EVS or MM traps (Li *et al.* 2016, Lühken *et al.* 2014), but the results have been dependent on multiple factors and varied from one study to other. It is important to consider, as pointed out by Brown *et al.* (2008), that differences between traps could affect the estimations of species abundance and composition.

Figure 1. Classic EVS mosquito trap baited with a container with CO_2 pellets.

Figure 2. BG sentinel trap.

Traditional surveillance methods have two main limitations. The first is the cost in terms of time and professionals involved in the surveillance (trap placement, sample collection and transport of the sample to the laboratory for the counting and identification of captured mosquitoes). The second limitation is the inevitable time lag between the moment that the trap is placed in the field and the moment the sample is collected. This lag can be crucial, potentially resulting in the dynamics of mosquito populations in the field not being accurately and timely monitored (Focks 2003).

In this scenario, artificial intelligence (AI) is forging a path in improving traditional entomological surveillance methods by generating new techniques for the automated remote monitoring of mosquito populations. These new approaches include the emergence of automated electronic devices which remotely classify mosquitoes based on the analysis of their flight pattern (Potamitis 2014, Santos *et al.* 2019).

In addition, the use of the 'Internet of Things' (IoT) is enabling that the information collected remotely in the field could be sent wirelessly to a cloud server in real time (Eliopoulus *et al.* 2018, Geier *et al.* 2016, Potamitis *et al.* 2017). Thus, eliminating the gap between trap installation and collection, representing mosquito population dynamics much more accurately.

New technological approaches for remote mosquito surveillance through the perspective of artificial intelligence

Acoustic sensing technology

Mosquito flight tones have been extensively studied since the first half of the 20th century, mainly through the use of acoustic methods such as microphones (Kahn and Offenhauser 1949). Mosquito flights produce a tone as a side effect of wing movement. This tone is also a communication signal that is frequency-modulated during courtship and can be detected by other mosquitoes thanks to certain properties of their antennae including Johnston's organ at the base of each antenna (Cator *et al.* 2009, Gibson *et al.* 2010). Rapid frequency modulation flight in males occurs as a response to female wing beat frequency and is likely to represent a pre-copulatory controlled flight to maintain a close-range position while attempting to seize and engage terminalia with the female (Simões *et al.* 2016). Females have the ability to reject or accept the male mating attempt. In the event that the interaction between male-female pairs is successful, copulation will take place preceded by an acoustic harmonic convergence (Aldersley and Cator 2019, Aldersley *et al.* 2016).

With these acoustic properties in mind, entomologists have been pursuing the control of mosquitoes by means of sound traps for many decades (Kahn and Offenhauser 1949) and continue to do so (Diabate and Tripet 2015, Rohde *et al.* 2019). Sound traps, such as the Sound Gravid Aedes Trap (SGAT), the Male Aedes Sound Trap (MAST) (Staunton *et al.* 2020a), or other modified commercial traps with an acoustic basis, are nowadays being used as cost-effective alternatives for field use in areas with sterile male mosquito rear-and-release programs (Johnson and Ritchie 2016, Rohde *et al.* 2019, Staunton *et al.* 2020b).

The acoustic detection of insects is a highly active research field, especially in its application to food crops and stored grain pests (Eliopoulos *et al.* 2016, Hagstrum *et al.* 2012, Potamitis *et al.* 2009) but also with respect to pests of medical importance, such as mosquitoes (Salim *et al.* 2017, Vasconcelos *et al.* 2019). In recent years, so-called deep learning techniques have become widely used in bioacoustic classification tasks based on the analysis of mosquito wing beat frequency. However, since mosquitoes from different species can actually have overlapping frequency distributions, it seems insufficient to use the fundamental wing beat frequency as the sole distinguishing characteristic between species (Chen *et al.* 2014). To improve the classification method, metadata such as time or place of recording can be used as additional features to differentiate between mosquitoes with varying circadian activity or geographic distribution.

Current approaches for mosquito wing-beat analysis and classification through acoustic sensors include the use of mobile phones as an easily available tool for entomological surveillance (Fernandes *et al.* 2020, Li *et al.* 2017, Mukundarajan *et al.* 2017). Mobile phones offer the advantage of automatically registering time and location stamps for acoustic data and allow the collection of other metadata such as photographs which can support identification. Studies based on mobile phone-based bioacoustics demonstrate that even low-cost smartphones are capable of accurately recording mosquito wing-beat frequencies, enabling continuous and large-scale data mapping which can be particularly useful in resource-constrained areas (Mukundarajan *et al.* 2017). In this sense, there are some open data platforms that rely on the participation of non-expert volunteers to record the wing-beat sound of the mosquitoes. Two of the most popular ones are 'ABUZZ' (Mukandarajan *et al.* 2019) and 'Humbug Zooniverse' (Kiskin *et al.* 2020).

The inconvenience of acoustic methods is the limitation to the quality of the microphone recordings of the insects in field conditions. Many mosquito bioacoustics experiments are undertaken in unnatural conditions with tethered individuals or in acoustically isolated spaces, thus leading to difficulties to apply these models in in field conditions (Arthur *et al.* 2014). Given this difficulty in microphone-sourced field recordings, classification models based on machine learning algorithms commonly suffer from scarce and poor-quality data.

Chen *et al.* (2014) reported a 'lack of progress' in acoustic technology applied to the automatic classification of insects. This can be attributed to limitations of the microphones themselves. One such limitation is microphone sensitivity. The sound attenuates with the distance from the microphone according to an inverse squared law, which means that if an insect is flying three times more distant from the microphone, the sound intensity will drop to one ninth. When increasing the microphone sensitivity to mitigate this effect, any surrounding noise will saturate the signal. Filtering insect detection can then become a complex task, as well as requiring more system processing power. Besides, systems based on a microphone and recorder set spend the entire experiment running time making recordings, thus increasing power consumption.

The foremost challenges for acoustic sensing approaches are related to dealing with the problem of the signal-to-noise ratio of recorded audio and power consumption. As a result, optical approaches for remote sensing and automatic classification have gained in popularity as they offer significant performance advantages (Potamitis and Rigakis 2015, Santos *et al.* 2018, 2019).

Optical sensing technology

Optical technology for mosquito wing beat analyses dates back to the second half of the 20th century when the first photoelectric cell was discovered to detect the light modulation of a flying insect crossing its field of detection (Richards 1955). This was the starting point for the implementation of numerous studies on the use of optical sensors to monitor mosquito flight patterns which continue to the present day (Gibson *et al.* 2010, Kirkeby *et al.* 2016, Ouyang *et al.* 2015, Potamitis 2014, Potamitis and Rigakis 2016a).

The diverse light source options for optical sensing include laser and LED (light emitting diodes). Potamitis and Rigakis (2015) developed a novel noise-robust optical sensor to record insect wing beats and analysed the recording performance of both types of light sources, comparing them to the recordings of an acoustic sensor. The results showed that both performed as well or even better than the acoustical sensing approach in any ambient light condition. Unlike acoustic sensors, optoelectronic sensors only record when triggered by flying insects, allowing large savings in power consumption. In addition, optoelectronic sensors are capable of modulating the optical signal at high frequencies, thus eliminating major optical interference sources and increasing sensor efficiency without further data processing requirements (Santos *et al.* 2018).

Optical sensors basically comprise an optical emitter (a laser beam or a LED array) and an optical receiver (a phototransistor, mainly photodiodes) creating a FOV (field of view). When an insect crosses the FOV, fluctuations in light intensity (caused by the partial occlusion of the light from the wing's movement) are perceived by the optical receiver. The signal containing information on the detected insect's wing beat frequency is then amplified, filtered, and demodulated in an audio signal (Batista *et al.* 2011, Potamitis and Rigakis 2016b). The conversion of the optical signal into audio data allows comparison of the results obtained with those available in the literature for acoustic systems.

The practical applications of these new findings involve extending the use of optical sensors from laboratory tests to the production of massive datasets and the creation of smart insect traps that can count, recognise, and alert for the presence of insects of economic and public health importance (Potamitis *et al.* 2018). Novel optoelectronic sensor prototypes are being trained with several machine learning algorithms, mainly Bayesian classifiers, to learn how to distinguish between mosquito species and mosquito gender (male and female) based on their wing beat frequency (Batista *et al.* 2011, Genoud *et al.* 2018, 2019; Ouyang 2015; Potamitis and Rigakis 2016b). While high accuracy values in gender discrimination are now commonly obtained, classification to species level is still challenging (Genoud *et al.* 2018), although the use of deep learning techniques has shown promising levels of precision (Fanioudakis *et al.* 2018).

The biggest difficulty appears when trying to distinguish two different mosquito species from the same genus as they may have overlapping frequency spectrums. This suggests that the fundamental wing beat frequency alone, although it may be sufficient to distinguish the mosquito genus or gender, it may be insufficient on its own to properly classify mosquito species. This inefficacy will be even more apparent in the context of field measurements, where plenty of mosquito species, Diptera and other insects may be present. A common way to improve identification accuracy is to add other predictor variables in addition to fundamental wing beat frequency (Batista *et al.* 2011, Chen *et al.* 2014, Genoud *et al.* 2019). For instance, Genoud *et al.* (2019) proposed the use of the depolarisation ratio of the mosquito body together with the wing-beat frequency to distinguish gravid from non-gravid females, which reported high accuracy results.

Another option to increase the accuracy of automated taxonomical classification of mosquitoes in field studies may be the use metadata (Chen *et al.* 2014): meteorological features (temperature, humidity, and air pressure), spatiotemporal features (distance from freshwater, land cover type, human/livestock population density, local agricultural type, time of year, time of day, etc.) and circadian rhythms. Certain species are more adapted to survive in particular environmental conditions, e.g. many mosquitoes are native to tropical and subtropical regions, where the climate is typically warm and wet. The ambient temperature can be determinant in insect classification since it influences insect metabolism, leading to an increase in the wing beat frequency. Villarreal *et al.* (2017) reported an increase of 8-13 Hz per degree Celsius (°C) in females of *Aedes aegypti*, revealing a highly dependent relationship between these factors. Circadian rhythm is also an important feature to be considered since mosquitoes have different peaks of activity throughout the day which can be of help to distinguish between species. However, circadian rhythm cannot be used without at least a rough estimate of the population of each considered species (Genoud *et al.* 2019). If a species with a small population has an activity peak at the same time as another with a much larger population but with lower activity, although their probability of interaction with the sensing instrument may be equal, the classification system will consider the former to be much more likely, thus inducing a bias in the results.

New optoelectronic devices for remote sensing include, in addition to insect counts and classification, the use of IoT technology. This allows that the entire information that is being registered remotely in the field, is also being transmitted wirelessly to a central monitoring agency in real time for risk assessment analysis. In this way, novel optoelectronic sensors can be self-organised in networks that collectively report data at local, regional, country, continental, and global scales. The emergence of so-called e-traps has the potential to profoundly impact entomological surveillance and pest control (Potamitis *et al.* 2017).

Smart trap technology

Novel smart traps entail the possibility of automating everything that is still presently done manually (collecting insect information in the field, processing that information, and sending it to vector control technicians) thanks to the use of IoT technology. The development of IoT solutions using conventional approaches is complex and time consuming due to the lack of common architectures and languages, and the widespread use of non-standard, proprietary interfaces and sensor data formats. Numerous developers, companies and R&D groups have been using state-of-the art commercial platforms like Arduino (Italy), Raspberry Pi (UK) or BeagleBone (USA), which are capable of prototyping straightforward sensor applications with low technology readiness levels (TRL) of between 1 and 4. However, such platforms may be insufficient if advances are to be made to TRL 5 prototyping and above, especially if dealing with sensors that are not off-the-shelf. This implies that off-the-shelf platforms offer limitations to reach TRL 9 (go-to-market), where manufacturers will be fighting issues of functionality, cost, power consumption, scalability, margin, manufacturability, testability, packaging, mechanical robustness and working conditions (e.g. temperature, humidity), etc.

To address this, IRIDEON (Spain) has developed SENSCAPE®, a disruptive, modular, standards-based framework for the development of fast IoT time-to-market solutions. There are several advantages to developing an IoT application with SENSCAPE: (1) ready-made hardware platforms – static and mobile; (2) standards based, (3) interoperability; (4) scalability; (5) low power consumption; (6) reduced costs; (7) smartphone integration; (8) customisation and (9) cloud-ready. The general idea is to use SENSCAPE® as the platform to combine a sensor capable of capturing physical information from flying insect, with two emerging disruptive technologies: IoT and AI.

The IoT refers to systems of physical devices that receive and transfer data through wireless networks without human intervention, while AI refers to the combination of algorithms developed to have machines reasoning like human beings. The combination of these various elements could lead to a solution where each trap acts as an interconnected device that can remotely analyse each captured flying insect, just as a professional entomologist would do.

For the moment, only one optical sensor product designed for the remote monitoring of mosquito populations is commercially available, the Biogents BG-Counter (Germany) (Geier *et al.* 2016). This device is able to distinguish between mosquitoes and other different insects, and to count mosquitoes, but does not provide further information on mosquito species, sex or other attributes. In parallel, there is another optoelectronic sensor prototype that has been created by IRIDEON (Spain), which is already capable of distinguishing between species, sex and age of mosquitoes in laboratory conditions (Brosa 2018). The mosquito sensor is an optoelectronic device comprising an emitter, an array of LEDs, and an array of phototransistors acting as photoreceptors connected in parallel. This optical setup generates a light field. The sensor constantly captures the input from the sensor but only processes the samples when a triggering event occurs, i.e. when there is a perturbation of the light field. Optical sensor, microprocessor, and wireless communications are integrated into the electronics module.

Smart trap stations can be deployed as a wireless sensor network (WSN) with bidirectional management of data between sensors and a cloud application framework. When an insect is drawn into the trap equipped with a sensor, its characteristic wing flapping modulates the light field. Captured signals are sampled at a rate sufficient to resolve the fundamental frequency of the wing-flap as well as several overtones. The light field is also perturbed by other physical elements associated to the flying insect: the kinetics of flight (speed, direction, and trajectory) and morphology (body/wing size and shape). Each of these physical elements of flying insects that cause a perturbation of the light field leads to a species-specific signature. The signal of this signature is filtered, amplified, acquired and processed using a combination of AI methods (e.g. rule-based systems, genetic algorithms, artificial neural networks and fuzzy models). Depending on the tests performed, these methods can be used to count each event that perturbs the light volume, determine if the event is caused by a flying insect, analyse if the flying insect is a mosquito or not, classify the genus of the insect, identify the species, identify the sex and estimate the age in days (Figures 3 and 4).

These assets have been benchmarked by experts and judged to be at TRL 7 for genus (accuracy 90-92%), species (accuracy 75-80%) and sex classification (accuracy 93-99%) of *Aedes albopictus*, *Ae. aegypti* and *Culex pipiens*. The sensor also achieved TRL 5 for age classification of each of these species (accuracy 61-95%), giving an overall TRL 6 (Brosa, 2018). Further work is being done to improve the overall accuracy of the solution to reach TRL 9 by 2022.

How intelligent traps can improve mosquito monitoring and arbovirus control programs

Integrated pest management (IPM) relies on the accuracy of pest population monitoring (Freier and Boller 2009). Without gathering information of population dynamics, and related ecological factors, it is almost impossible to execute an appropriate control at the right place and time. Mosquitoes are usually spread across large areas and boundaries, and the use of traditional surveillance methods which are strongly dependent on human labour is unsuitable for efficient large-scale monitoring (BIPRO 2009). Fully automated remote monitoring could be the key in this context.

Earth observation service for preventive control of insect disease vectors – the VECTRACK project

Obtaining high quality field information is notoriously costly and time consuming. The amount of money required can significantly be reduced by combining cost-efficient sampling strategies,

Figure 3. Vector control technician installing a trap with IRIDEON's smart mosquito sensor.

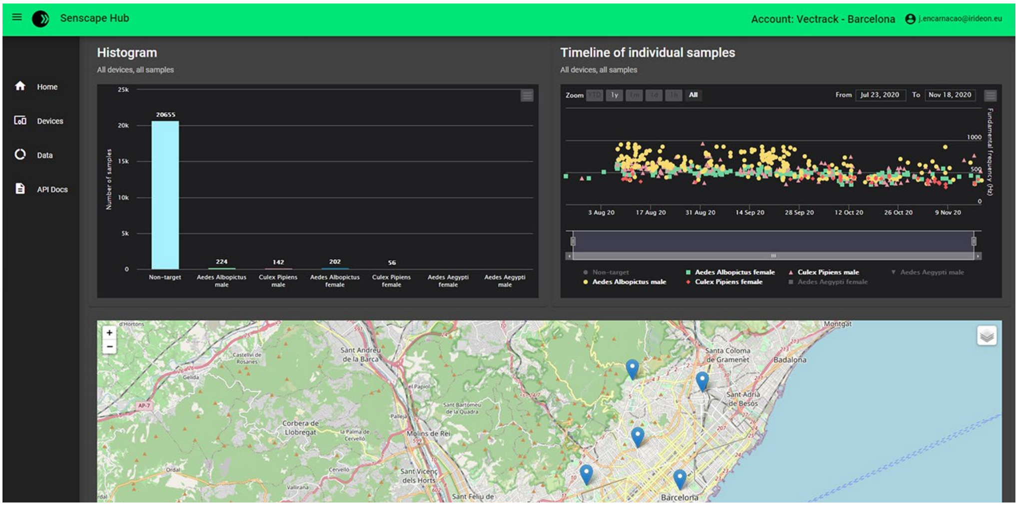

Figure 4. Dashboard of the software cloud application of the smart mosquito sensor.

remote sensing, and spatial modelling techniques to compute risk maps of vector presence and abundance, as well as maps indicating high-risk zones for the establishment of exotic species at local or regional level. Such maps could then serve as a basis for targeted surveillance and VBD risk assessments. To address this, IRIDEON is heading a Horizon 2020 (H2020) project called VECTRACK, in collaboration with AVIA-GIS (Belgium), the IRTA-CReSA research institute (Spain) and the public health institute CEVDI/INSA (Portugal). VECTRACK constitutes a novel and unique opportunity, integrating the added value of Earth observation (EO), spatial-positioning and information and communications technology (ICT) technologies: Copernicus data + operational vector mapping with spatial modelling + IoT ground sensors + IoT smart mosquito traps + IoT interoperable disease vector data cloud application. The proposed innovation is a service platform for which Copernicus is a critical part of the solution. The main objective is to develop and validate a new Copernicus-based EO service to monitor disease vectors, associated to a novel ground wireless sensor network comprising miniaturised nodes measuring micro-environmental data (T °C, %RH, etc.), together with a smart trap station acting as a gateway.

Earth observation platform can measure land surface temperature and vegetation, which act as the main drivers of vector population (C3S 2020). Given the importance of the evolution of the meteorological parameters, the technical requirements for these satellites are: (1) high temporal resolution (1 day); (2) medium spatial resolution (1 km); and (3) measurement in the visible/near infrared part of the electromagnetic spectrum for derivation of vegetation indices and in thermal infrared for temperature.

In this context, it is important to mention the contribution of AVIA-GIS in their development of VECMAP, a seamless system and service that integrates the entire process of producing risk maps into a single package that supports all the steps required to map and model, at various scales, the distribution of vectors and to plan surveillance and control programs. This system provides all the satellite data required to obtain the risk maps, however is limited by the fact that it uses data from periodical manual trap inspections. This value proposition is strengthened by IRIDEON's smart IoT ground sensors, deployed in the field integrated with standard commercial mosquito traps. With the combination of all approaches, it is finally possible to remotely and automatically acquire near real-time ground data on mosquito counts, sex, species, age and local micro-environmental parameters. This data is invaluable as an automatic and direct input to feed mosquito-borne epidemic models.

Future approaches

With the use of novel smart traps, new challenges will appear; the automated identification of different mosquito species should be improved to the same level as when it is performed by a skilled entomologist and should be supervised until this degree of accuracy is reached. New maintenance and logistic protocols will need to be developed, as traps will go from being mobile and temporary to fixed and permanent.

With new methodologies, surveillance and control programs can be significantly affected as they require important scientific and logistic efforts for the management of large amounts of mosquito traps and collected samples. With the use of remote monitoring systems, once the system has been developed, these efforts can be redirected to other areas and most of the classification work would be done in an automatic way, but always with an accurate quality control system. The data will be completely interoperable and thus available for the automatic running of prediction models, the performance of risk assessments, the issuing of warnings and the undertaking of historical analyses of infested areas. In this way, vector control professionals could establish automatic surveillance programs with unprecedented accuracy and responsiveness, overcoming the labour costs of manual inspections. As a result, disease vector species will be detected earlier with greater precision, enabling improved control of outbreaks and a lower risk of disease transmission.

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12. Classic and novel tools for mosquito control worldwide

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Abstract

Mosquitoes (Culicidae) are at the centre of worldwide entomological research and control efforts primarily because of their medical importance as vectors of diseases, like malaria, dengue, Zika, Chikungunya, West Nile or Yellow fever. They are responsible for more than half a million deaths per year. Despite their role as vectors, culicids can also cause considerable nuisance like floodwater mosquitoes frequently create as they can reproduce in a short time in enormous numbers. The consequence is that outdoor activities in parks or recreation areas are not possible and this has a detrimentrous effect on touristic activities. The most successful approach for managing nuisance or vector mosquitoes is when an integrated vector mosquito management (IVM) is implemented in which all appropriate technologies and control techniques are used, to bring about a decline of target species populations in a cost effective and environmentally safe manner. The IVM strategy can include environmental management, physical, chemical, biological or genetical components. Environmental management means physical reduction of breeding resources, water management to create conditions unfavourable for mosquito breeding. Physical control includes the use of nets and surface layers to avoid vector contact or breathing by mosquito developing stages. Chemical control by using organochlorines, organophosphates, carbamates or pyrethroids is still the most frequently practised approach to combat mosquitoes but usually these chemicals are broad-spectrum products which can have also unwanted side effects on non-target organisms and on the biodiversity when they are used in ecological sensitive areas. Therefore, biological control aiming at the reduction of target populations by the use of predators, pathogens or toxins from microorganisms are nowadays more and more in focus of control operators. Especially the use of protein toxins such as from *Bacillus thuringiensis israelensis* or *Lysinibacillus sphaericus* provide efficient control of target organisms on the one hand and environmental safety on the other hand. The increased application of biological and microbiological methods or Insect growth regulators as well as genetic methods as the Sterile Insect technique (SIT) contribute to an environmentally friendly solution of the mosquito problems. New and improved techniques like the CRISPR (clustered regularly interspaced short palindromic repeats) as a mean of editing mosquito genomes to drive desirable gene constructs into mosquito population can help in future to avoid the transmission of human pathogens. The Geographic Information System (GIS) integrated with digital mobile collection systems supported by a Global Positioning System (GPS) and modern information-technology, can significantly contribute to improving the planning, realisation and documentation of mosquito control/management operations and allow a more effective effort to reducing mosquito-borne diseases. It is out of question that all strategies should involve the public to raise the awareness of people, e.g. for the control of invasive mosquitoes by community participation.

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Keywords: mosquito control, vector management, environmental health, insecticides, biological control, applied biotechnology, integrated pest management (IPM)

Introduction

Mosquitoes (Diptera: Culicidae) represent one of the most important family of arthropods in the worldwide transmission of pathogens to humans and animals (Becker *et al.* 2003, Taylor *et al.* 2010), being consequently one of the most important causes of mortality in developing countries. The fight against mosquitoes has ancient origins that date back to the times of Herodotus (V century BC) (Genchi and Pozio 2004) when it was aimed to reduce the nuisance caused by these insects (Swift 2008). Just from the end of the 19th century, with the discovery of the role of vector of heartworms and malarial plasmodium (Capanna 2006, Chernin 1983), the mosquito control strategies begin to take public health connotations. Many countries applied both chemical and mechanical methods, mainly through the use of the organic chlorinated insecticide DDT, and the remediation of marsh areas (Rahman 2013, Tognotti 2008), respectively.

The progressive increase in knowledge about pathogens transmitted by mosquitoes and the spread of alien species introduced in areas far from their origins in recent years, have required even greater attention from public health institutions. At this point, the implementation of mosquito control programs for the prevention of epidemic situations and for the improvement of the quality of life, are considered a priority in many countries (Bellini *et al.* 2011).

Besides the impact of mosquitoes on public health, these insects are also a great threat for wildlife. Many zoonoses caused by viruses and parasites are transmitted by mosquitoes. Avian populations are strongly affected by arbovirus like West Nile, Sindbis or Usutu Virus among many others. These arboviruses are mainly transmitted by *Culex* and *Culiseta* mosquitoes, and despite they have an African origin nowadays outbreaks of these pathogens can be seen on bird populations in different continents due to bird migration processes and climate change conditions which favours virus replication in local mosquito populations (Buckley *et al*. 2003, Ling *et al.* 2019, Reusken *et al*. 2011). Avian malaria is a parasitic disease caused by Apicomplexa species belonging to the genera *Plasmodium* and *Hemoproteus* which can be also transmitted by mosquitoes, once again *Culex* and *Culiseta* species as principal vectors due to their ornithophilic behaviour. There is a wide range of symptoms that these parasites can provoke on birds, from asymptomatic infections to serious affectations characterised by fever, diarrhoea, and anaemia. Even in some cases drastic declines on local bird populations have been described associated to avian malaria epizootics (Van Riper *et al.* 1986). From the point of view of mosquito-borne zoonoses that can seriously affect wildlife, Dirofilariasis (basically associated to *Dirofilaria immitis* and *Dirofilaria repens*) and sylvatic Yellow Fever epizootics on non-human primates can be also highlighted.

Mosquito control can be achieved through the application of direct control techniques which affects the target organisms increasing their mortality, or alternatively through indirect control techniques which affects their reproduction rate (Figure 1).

The mosquito prevention and control techniques can be classified by the following typologies according to their effects, approaches and origin of the control tools (Table 1):

Environmental. It includes indirect control methods which predominantly try to make a specific vulnerable territory (e.g. crop area with permanent stagnant water) unsuitable for the development of mosquitoes, after the application of several environmental changes which, when linked to the

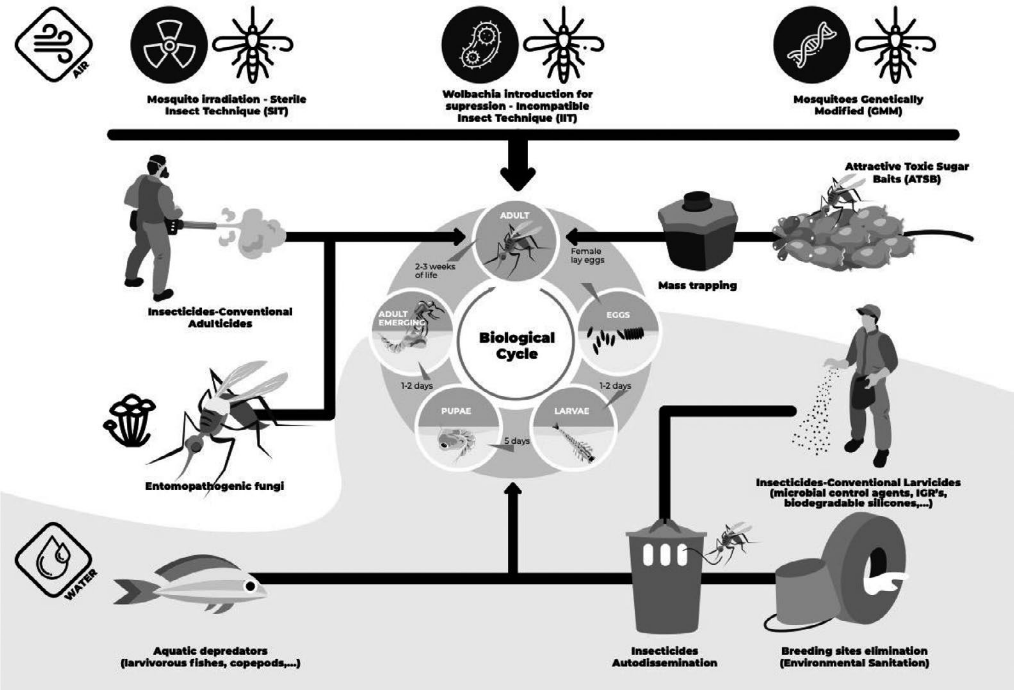

Figure 1. State of the art of different mosquito control strategies, specifying their impact on adult or juvenile life stages.

Table 1. Prevalent effect of different mosquito control strategies.[1]

Type of struggle	Mortality increasing	Reduction of reproduction rate	Feeding suppression
Environmental	–	+	–
Physical	–	–	+
Mechanical	–	+	+
Biological	+	–	–
Biotechnology	–	+	–
Chemical	+	–	+
Educational	+	+	–
Legislative	+	+	–

[1] Positive effect (+), non-effect (–).

cultivation cycles, are named 'agronomical'. A typical example of environmental control strategy is the one carried out in Italy in the early decades of the 1900s to suppress the proliferation of *Anopheles* Meigen, 1818 (Majori and Napolitani, 2010). The reduction of the potential breeding sites are included in the environmental method (Dutto *et al.,* 2012).

Physical. It is an indirect method that aimed to transform habitats potentially colonised by mosquitoes in non-suitable ones through the modification of environmental conditions.

Mechanical. It probably represents the oldest mosquito fighting strategy, essentially based in the interposition of physic barriers (e.g. mosquito nets) that prevent the access of mosquitoes to oviposition sites, or host for blood feeding. Subsequently, substances capable of blocking the gaseous exchanges of the larvae and pupae with the atmosphere (e.g. silicones, oils) have been developed in last decades, provoking asphyxiation processes in immature aquatic stages.

Biological. It involves the use of natural predators (e.g. fishes, copepods), microorganisms (e.g. bacteria, fungi) or toxins linked to these microorganisms.

Biotechnology. The strong advances of molecular manipulation techniques has allowed the use of genetically modified mosquitoes (GMM), mosquitoes irradiation to achieve sterile insect techniques (SIT) or the introduction of endosymbionts like *Wolbachia* to promote the employment of incompatible insect techniques (IIT). Most of these methods try to suppress mosquito populations reducing their fertility or lifespan.

Chemical. Together with the mechanical methods, chemical approaches represent one of the oldest control methods. Chemical control plays a crucial role in the control of mosquitoes both at the larval and adult stage, and it is essentially based on the dispersion of chemical synthetic substances capable to cause direct lethality or repellency on target insects.

Educational. The engagement of citizens and local communities in the fight against mosquitoes is essential, particularly in domestic habitats where many species of mosquitoes can find adequate breeding sites. Door to door strategies and informative campaigns in towns, residential areas and schools, have been implemented in many countries in order to reduce the infestation levels of *Aedes* Meigen, 1818 mosquitoes which can cause important outbreaks of arboviruses.

Legislative. The adoption of legal regulatory rules that oblige the implementation of individual and collective preventive measures to fight against mosquitoes is currently considered as an important issue in any local mosquito control program.

The joint application of several techniques, in association with appropriate surveillance and monitoring programs, allows the development of integrated mosquito management programs (IMMP) which improve efficacy, efficiency and safety of interventions.

Chemical control

Adulticiding

In an IMMP, adulticidal applications should be performed just in specific situations to complement larvicidal interventions, or also to manage situations of mosquito-borne diseases (MBD) epidemics. The use of the adulticides as unique intervention strategy has been demonstrated ineffective to maintain low levels of mosquitoes in a determined area. Moreover, insecticides used to kill adults (mostly pyrethroids) in outdoors have a high environmental impact (Hénault-Ethier 2015).

The adulticidal fight is purely chemical and is implemented through the environmental dispersion of molecules with killing insecticidal activity and can be carried out both indoors and outdoors. The ideal adulticiding should aim at the treatment of resting sites in the hours of inactivity of the target species and taking into account the favourable environmental conditions of each time. Adulticidal treatments can be divided into (Romi *et al.* 2009):

Spatial treatments in outdoor conditions, generally concerning vegetation of green areas, heaps of materials (e.g. demolition) or ruins of buildings in degraded urban areas. This kind of applications will depend on resting behaviour of the target species.

Residual treatment in indoor conditions, usually restricted to interior and semi-interior wall treatments, concerning indoor environments of buildings.

Historically, the active ingredient most used for the control of mosquitoes and many other vector insects has been dichloro diphenyl trichloroethane (DDT). This active substance was endowed with a high insecticidal capacity and low acute toxicity, but at the same time had an unacceptable ecotoxicological profile. DDT has currently been banned since the 1970s in Europe and the United States of America, although it is currently still in use in many developing countries despite global production and use being undergoing modest decline since stipulation of the Stockholm Convention (Van den Berg *et al.* 2017).

The active compounds currently most in use belong to the pyrethroid family which constitutes 25% of the world market of insecticides (Soderlund *et al.* 2002). The photostable second generation pyrethroids (e.g. deltamethrin, cypermethrin, cyfluthrin) are mainly used in open environments, while the first generation non-photostable pyrethroids (e.g. allethrin, tetramethrin) are used both for repellent and killing purposes in premises (Scirocchi 1998).

Pyrethroids act by contact and ingestion, being the contact mode action facilitated by their liposolubility which provokes an easy absorption by the cuticular waxes of insects. The mechanism of action is due to the depolarisation of the neuron membrane by action on the sodium channel which is followed by the total block of nerve transmission. In the case of pyrethroids with a hydrocyanic group in the molecule, the block affects the central nervous system (CNS) and is irreversible. Toxicity towards homeothermic animals (with some exception, e.g. cats) is low. From the environmental point of view, since they are insecticides without any selectivity, the use must be carefully evaluated before carrying out a treatment in green areas (Bianco 2015).

Finally, it is necessary to remind and emphasise that pyrethroids can give rise to resistant populations (Brengues *et al.* 2003, Mint Mohamed Lemine *et al.* 2018) and it is therefore advisable, in order to avoid the onset of this phenomenon, to make a careful evaluation of the active ingredients before treatment and investigate any post-treatment failures with laboratory efficacy tests. The use of molecules belonging to other families (e.g. carbamates and organophosphorus) are no longer authorised in several continents for space treatments for toxicological and ecotoxicological reasons.

Larviciding

The larvicidal interventions are focused on the sites where larvae are developing or can potentially do it. The agronomical and mechanical strategies consist in the elimination of water resources (e.g. drainage, tanks and containers for irrigation) or in preventing that mosquitoes can reach (e.g. lids to close buckets, mosquito nets) these sites suitable for eggs oviposition. The agronomic interventions generally affect large areas with repercussions on large portions of territory, while the mechanical interventions find more point-like applications even in private areas (e.g. gardens and parks) in urban environments. Another type of mechanical fight is the application, in the mosquito breeding sites, of non-polar substances, with a specific weight lower than water and with low surface tension. These substances cause the formation of a surface layer which prevents the larvae from breathing, causing death for asphyxiation. This strategy was also applied in the past century using mineral oils, with important consequences for the environment (Harwood and James 1979). In recent years, silicone substances as well as vegetable oils have been developed to get good results but without of such negative environmental impacts (Webb and Russell 2009).

Another group of substances capable of interfering with the pre-imaginal development of mosquito larvae are the insect growth regulators (IGR's). The IGRs most commonly employed for mosquito control can be divided into Cuticle Inhibitors (CuI) and juvenile hormone analogs (JHA). The CuI are substances belonging to the benzoylureas of which diflubenzuron is the most used for the control of immature culicids. These substances act mostly by ingestion on the preimaginal larvae stages, interfering with the cuticle formation process during the pre-imaginal stages. The mechanism of action is based on the inhibition, by the active ingredient, of the enzyme chitin-synthetase. The inhibition of this enzyme prevents the deposition of N-acetylglucosamine which is essential for the formation of chitin in the cuticle and the peritrophic membrane of the intestine. Death occurs by rupture of the integument during a moulting or metamorphosis process. Diflubenzuron also has an ovicidal action and in this case acts by penetration of the chorion following contact, in particular in the newly laid eggs; the ovicidal properties are not particularly important in the fight against mosquitoes, considering that many species lay dry and not directly in water. The IC do not show selectivity in the context of invertebrates and consequently are not suitable for application in water collections communicating with protected wetlands or environments where large biodiversity of invertebrates is present.

The JHA or juvenoids are substances that only in recent decades have been deeply used in the fight against mosquitoes and other dipterans. These substances act principally by penetration the cuticle or the chorion and the mechanism of action varies according to the specific substance. The way of action is a juvenilizing effect as substances similar to neotenin or juvenoid hormone with death determined as a result of biochemical alterations in the development cycle. In the case of analogues of neotenin (e.g. Methoprene) the presence of these substances in the hemolymph prevents ecdysone from activating, in the cells of the whole organism, those chromosomal tracts in which the structure of the adult is encoded. The consequence is to keep only the larval genome active. Some other active ingredients (e.g. Pyriproxyfen) also exert an inhibitory activity of neotenine esterase, consequently preventing phenomena of metamorphosis to adult stage. The insecticidal action can be observed starting several days after application and the presence of viable larvae or pupae within the treated foci does not indicate the absence of treatment. In such cases, to verify the effectiveness of the treatment it is necessary to sample the larvae and keep them until metamorphosis into an adult when dead pupae and pre-adults could be observed. They have little or no selective action among insects even if they are endowed with low toxicity towards vertebrate animals.

Undoubtedly, larviciding through the use of microbial agents is probably the strategy that has been more quickly developed and established, especially in developed countries. The high degree of efficacy and principally the environmentally friendly approach of these biological products, which are even causing specific mortality only in culicids in some cases, are strong arguments that support the increasing interest on these biological insecticides. However, the economic cost in product manufacturing compared with other kind of insecticides, is a major concern nowadays for their introduction at large scale in developing countries with low economic resources and possibilities. More information about this topic is provided in the section of biological control.

Finally, it is necessary to consider the possibilities of chemical control. This type of applications is practically in disuse in developed countries, while it is still used in developing countries strongly affected by mosquito-borne diseases (MBD). The applications are generally done spraying water collections with pyrethroid or organophosphorus products. The action is carried out very quickly by contact and ingestion and does not show any selectivity towards both invertebrates and heterothermic vertebrates, being particularly toxic for fishes. Other chemical tool occasionally employed for mosquito larvae control is the use of metallic copper in the breeding sites. The toxic action is related to Cu ions releasing and the metal oxidation compounds. The effectiveness of metallic copper is strongly influenced by the type of metal processing (braids, wires, etc.); killing is already observable starting from concentrations of copper metal >1 g/l (Della Torre *et al.* 1993), concentrations from 4-8 to 20 g/l of metallic copper cause high mortality rates that can even reach 100% with persistence of the toxic effect for months (Della Torre *et al.* 1993, Romi *et al.* 2000). It has recently been shown how the application of Cu metallic in spray formulation with dosage of 500 µg/kg determines 100% of mortality in 2 weeks (Becker *et al.* 2015).

The use of copper metal, even in domestic and public water collections (e.g. small containers), is a practice not recommended in order to avoid excessive dispersion of heavy metals and for regulatory problems (European biocide directive) related to the active ingredient (Bellini and Veronesi 2006). It is a practice that can be evaluated urgently for outbreaks of MBD in the absence or lack of other active ingredients.

Biological control

Biological control, in the broadest sense, is defined as the reduction of the target population by the use of predators, parasites, pathogens, competitors or toxins from microorganisms (Becker *et al.* 2020, Benelli *et al.* 2016, Huang *et al.* 2017). Biological control aims to reduce the target population to an 'acceptable' level and at the same time, to avoid adverse effects to the ecosystem. As far as mosquito control is concerned, biological control measures should integrate the protection of humans from mosquitoes with conservation of the biodiversity whilst avoiding toxicological and eco-toxicological effects (Becker and Lüthy 2017, Becker *et al.* 2020, Timmermann and Becker 2017). As a result, the regulatory power of the ecosystem is maintained by protecting the existing community of mosquito predators.

The use of beneficial organisms for the control of mosquitoes was first recognised in late 19th century, when attempts were made by introducing predators such as dragonflies (Lamborn 1890). However, mass breeding and successful introduction of predators such as hydra, flatworms, predacious insects or crustaceans, often introduces a range of problems. However, such problems did not occur, or only to a limited extent, with the use of fish such as the mosquito fish, *Gambusia affinis* (Baird and Girard, 1853) (western mosquitofish) and *Gambusia holbrooki* Girard, 1859 (eastern mosquitofish), which were successfully introduced into many countries to control mosquito larvae in the early 1900s (Bellini *et al.* 1994, Chandra *et al.* 2008, Legner 1995, Walton 2007).

With the discovery and large-scale use of synthetic insecticides in the 1940s and 1950s, biological control of mosquitoes was unfortunately no longer considered to be an important method. However, the initial euphoria that greeted the success of synthetic insecticides rapidly dissipated as resistance subsequently developed within the target populations. Moreover, despite the beneficial effects of traditional insecticides, they also often have unwanted characteristics, such as their non-selectivity which frequently causes ecological damage. As public awareness of environmental issues increased, regulations controlling the application of chemicals were tightly regulated. As a result, a renaissance of the biological control of mosquitoes took precedence in the 1960s and 1970s. By 1964, Jenkins had already listed more than 1,500 parasites, pathogens and predators as potential candidates for biological control. Today, the literature on mosquito antagonists is immense (Becker *et al.* 2020, Davidson 2012, Lacey 2017, Legner 1995, Quiroz-Martínez and Rodríguez-Castro 2007).

One of the major advantages of biological control measures is that existing predators are conserved, which will then assist the control effort by preying upon newly-hatched mosquito larvae after the control operation, thereby, considerably enhancing the efficacy of the current control measures. By promoting the conservation of existing populations of predators, parasites or pathogens, there are two major strategies for the augmentation of populations of mosquito antagonists (Becker *et al.* 2020, Lacey 2017).

Inoculation refers to the release of small numbers of predators, parasites or pathogens into the habitat of the target organisms. The antagonists become established, they reproduce and multiply under favourable living conditions in the new habitat, resulting in a sustained suppression of the target population (Walton 2007).

Inundation means the release of an overwhelming number of predators, parasites, pathogens or their toxins into the mosquito habitat. Such mass release of organisms or applied pathogens (toxins) can have an immediate effect through a significant reduction of the target population. For instance, inundative control is successfully practiced with microbial pathogens which are produced in artificial cultures, e.g. *Bacillus thuringiensis israelensis* (Bti) and *Lysinibacillus sphaericus* (Lsph). Only rarely, do the antagonists become established in the habitat, for example Lsp is able to recycle under certain conditions (Becker *et al.* 1995, Lacey 2017).

A prerequisite for the successful use of predators, parasites or pathogens, is precise knowledge of the biology of the antagonist in question and its interaction with the ecosystem. For example, the introduction of foreign faunal elements as predators, risks damaging or displacing existing populations of predators. For instance, introduced fish may reduce numbers of aquatic insects, crustaceans or amphibians which would otherwise be effective predators of mosquito larvae. Rare indigenous species which do not feed on mosquito larvae may also be endangered. A thorough understanding of predator/prey or parasite/host relationships is therefore of fundamental importance for the successful and ecologically sound use of antagonists. As a result of more than 100 mill. years of evolution mosquito species are able to inhabit very different habitats, and they have developed various life strategies by adapting to habitats with very different abiotic and biotic conditions. Antagonists can only successfully reduce a target population if their own life strategy is adapted to the target population. Here some examples of the efficiency of predators, parasites and pathogens are given.

Predators

In general, predators of the immature mosquito stages are more effective than predators of the adults. As a rule, mosquito larvae and pupae are concentrated at their breeding sites and are more easily available to predators than the widely dispersed adults. Moreover, adult mosquitoes evade many predators as they are mostly nocturnal. Mosquitoes have the characteristics of typical r-strategists (meaning, a high rate of reproduction and a relatively short life cycle). Predators are particularly effective if they have a similarly high rate of reproduction and/or a high rate of feeding, like fish (Becker *et al.* 2020). Macro-organisms such as fish have been used for decades as biological control tools in many mosquito controls programs. However, fish and other predators have specific ecological requirements and can only be used where their preferred living conditions are met. The life cycle of the predator is frequently not adapted to that of the target organism so that it is unable on its own to bring about an effective reduction of the target population. Mass rearing and release of the predators or parasites is often expensive or even impossible. This limits their large-scale use in a number of specific habitats. Special attention has therefore been given to the search for microbial control agents such as Bti (Davidson 2012).

Amongst the vertebrates, fish are the most effective predators of mosquito developing stages and can be even be used in the fight against malaria (Louca *et al.* 2009). The best known fish species is the mosquito fish *G. affinis* or the guppy *Poecilia reticulata* Peters, 1859. In the United States, mosquito fish are commonly bred by mosquito abatement districts and selectively released for control in an integrated mosquito management (IMM) program. However, before a non-indiginous organisms is released its prey-selectivity and their benefit as predator in relation to their environmental damage to the existing biota has to be studied. In general, native fish should be preferred which usually don't constitute a risk to the existing biota. Feeding rates of cyprinids can exceed several hundred fourth instar mosquito larvae (Becker *et al.* 2020).

Next to fish, amphibians can be effective natural occurring predators. Here the Urodela (newts) have to be mentioned which can also consume as adults several hundred fourth instar mosquito larvae/day. In contrast to urodelans, anurans (e.g. *Rana* species) have little effect as predators. In general birds and bats are not considered to be important regulators of mosquito populations, although mosquitoes can be a relevant source of food for some species (Becker *et al.* 2020).

Invertebrate predators

Countless invertebrates are known as predators of mosquitoes especially of the larvae. The biology and importance of the predators have been investigated in numerous studies (Becker *et al.* 2020, Dida *et al.* 2015, Service 1977). Although invertebrates have been shown to be effective predators of mosquitoes, they are seldom used in control programs due to the great difficulties and the high costs involved in mass rearing of these organisms. Nevertheless, their role as consumers of mosquitoes is beyond dispute. Mosquitoes can rarely develop in large numbers at breeding sites where predacious invertebrates are abundant.

Here are only a few examples of different groups of invertebrates and their importance as predators are given: *Chlorohydra viridissima* Pallas, 1776 (Coelenterata) ~10 larvae/day; flatworms (Turbellaria) *Mesostoma* sp. ~5 larvae/day; spiters and mites e.g. *Argyroneta aquatica* (Clerck, 1758) >20 larvae/day; Crustacea: amoung the crustaceans the copepods are very important predators of mosquito larvae. They can consume 1-2 first instar larvae/day. They can be introduced in artificial containers for the control of the two major vectors of dengue worldwide, namely *Aedes* *aegypti* (Linnaeus, 1762) and *Aedes albopictus* (Skuse, 1894). Amongst the insects the nymphs of dragonflies (Odonata) are very predacious. Water bugs such as *Notonecta* spp. Linnaeus, 1758 (Heteroptera) and water beetles, such as dytiscids (Coleoptera) are very important predators and even amongst the dipterans carnivorous larvae of Culicidae and Chaoboridae are particular predators of mosquito larvae e.g. species of the genus *Toxorhynchites* Theobald, 1901 in North America or *Mochlony culiciformis* or *Chaoborus* spp. in Europe. In aquatic ecosystems rich on aquatic organisms mosquitoes can usually not proliferate in great numbers due to the positive effect of predators (Becker *et al.* 2020).

Parasites and pathogens

The most important parasites of mosquitoes are the mermithid nematodes like *Romanomermis* spp. They occur mainly in water and have been tested as biological agents in various parts of the world (Lacey 2017). Unfortunately, these parasites did not become widely used because of difficulties with transportation, maintenance of the eggs and with the sensibility of the nematodes towards particular environmental conditions which made it difficult to establish them in mosquito breeding sites (Becker *et al.* 2020).

Bacteria

The discovery of the soil bacterium *Bacillus thuringiensis* subsp. *israelensis* (Bti) in the Negev desert of Israel in 1976 and of potent strains of *Lysinibacillus sphaericus* (Lsph) in have inaugurated a new chapter in the control of mosquitoes and blackflies (Becker and Margalit 1993, Becker *et al.* 2020, Mulla *et al.* 1990). The new subspecies of *B. thuringiensis* is highly toxic to larvae of most mosquito species and to blackfly larvae and to less extent to some members of other nematoceran families. New strains of Lsph, such as strain 2362 isolated from an adult blackfly in Nigeria (Weiser 1984) are much more potent than the first isolates and are particularly active against larvae of *Culex* species and *Anopheles gambiae*, the major malaria vector in Africa.

The discovery of these microbial control agents marked the breakthrough in biological control, because of the special abilities of these microbial agents. Their protein crystals are highly toxic to target organisms and extremely environmentally safe. Mass production of the bacteria, the availability of efficient formulations and the easy handling of the formulated products make microbial control tools a successful new weapon against nuisance and vector mosquitoes.

Biological control in the context of Nature Reserves, Zoos and other areas with protected wildlife

Dispose of tools and strategies to reduce mosquito populations without having impact on the rest of biocenosis is particularly important in protected territories where wildlife should be conserved. Protected wetlands are good example of these environments where mosquito control strategies must be carefully analysed in terms of cost-benefit before to be applied. The basis of these IMMP in wetlands should be related to the employment of non-chemical larvicides (Martinou *et al.* 2020), together with the habitats management for mosquito source reduction, as well as the introduction of natural predators (always local species without collateral impact on the rest of the fauna). This is particularly interesting since wetland creation, conservation or restoration projects often ignore possible impacts posed by mosquito population dynamics, mosquito-borne pathogens, nor do they always include mosquito management plans (Willott, 2004).

According to different institutions involved in Mosquito Management on Wildlife Refuges (US Fish and Wildlife Service, 2018), larvicides are generally preferred over mosquito adulticides in protected areas for several reasons:

a. Prevention: larviciding means the real prevention since the use of mosquito larvicides prevents the appearance of the blood feeding adults, which represent the nuisance stage of the cycle.
b. Residuality: mosquito larvicides can provide up to a month of control (3-5 weeks), rather than the few hours or days provided by fogging with adulticides.
c. Ecotoxicology: the commonly used mosquito larvicides are less toxic than the adulticides and are applied in such a way that there is much less non-target fauna exposure.
d. Cost-benefit balance: mosquito larvicides generally are applied to smaller areas than are adulticides.

Other key areas or territories where IMMP could have a crucial role in animal health protection are urban zoos (Quintavalle Pastorino *et al.*, 2015). Cases of mosquito-borne diseases like avian malaria (Grim *et al.,* 2004; Martínez-de la Puente *et al.,* 2020), Eastern Equine Encephalitis Virus (Tuttle *et al.*, 2005), West Nile Virus (Jett & Ventre, 2012), Usutu Virus (Weissenböck *et al.*, 2002) and Dirofilariasis (Sano *et al.*, 2005) have been documented in zoos. In these zoological facilities, once again the selective employment of biolarvicides and proper management of stagnant waters in order to reduce mosquito larval biotopes are essential environmental actions from the point of view of animal health.

Biotechnology and innovative tools for mosquito control

The need to explore new complementary and innovative mosquito control strategies and tools, beyond the traditional ones (insecticides for both adulticiding and larviciding interventions, and breeding sites removal through 'door to door' programs and citizen awareness projects) is widely recognised since relevant nuisances and/or mosquito-borne diseases are still present in the vast majority of countries from all over the world. This search for new alternatives in mosquito control is particularly urgent in urban environments where current control measures are highly ineffective in many countries due to the large availability of cryptic mosquito larval breeding sites, most of them in private areas where mosquito control interventions are difficult to implement by public agencies. Moreover, the increasing evidence in relation to chemical insecticides resistance phenomena in mosquitoes emphasises even more the need to evaluate alternatives for effective and efficient mosquito control programs. In recent years a lot of effort has been done in order to develop strategies like genetically modified mosquitoes (GMO), sterile insect technique (SIT) through mosquito irradiation, mosquito populations suppression employing the incompatible insect technique (IIT) by means of *Wolbachia* manipulation and introduction, or effective attractive toxic sugar baits (ATSB), among others. All these approaches aim to complement traditional vector control plans mostly based on chemical or biological insecticides applications with the last goal to implement integrative mosquito management Programs (IMMP).

Genetically modified mosquitoes

As occur with other non-GM methods, such as *Wolbachia*, there two major theoretical types of effects in nature that we can achieve when GMM are released in the field. First one is called 'population suppression' and aims to reduce or suppress mosquito population in order to minimise the impact of the species either simply in terms of nuisances due to mosquito bites or in relation to pathogens transmission. GMM can achieve this in several ways, like provoking biasing against the development of female progeny (sex-ratio distortion), reducing female fertility, introducing a mechanism that incapacitates or kills young female mosquitoes, and even some methods to shorten significantly the lifespan of female mosquitoes (WHO 2004). The second one is named 'population replacement' and has the focus on vector competence shortening by means of reduce the inherent ability of individual mosquitoes to transmit a given pathogen. In case of GMM the introduction of engineered DNA and/or the manipulation of endogenous genes inhibit pathogen replication within the mosquitoes, making them refractory to transmission (WHO 2004). Accurate releases of these GMM try to provoke changes in local wild populations, 'replacing' their inherent ability to spread the targeted pathogen with a reduced or eliminated transmission capability.

Several studies and field releases of *Ae. aegypti* and *An. gambiae* GMM, major vectors of dengue and malaria, have been conducted in recent years in several territories of Asia, America and Africa (Beisel and Ganle 2019, Carvalho *et al.* 2015, Subramaniam *et al.* 2012). Some of them showpromising results in terms of mosquito populations reduction (Carvalho *et al.* 2015, Gorman *et al.* 2016). However, there is a strong debate in the scientific and ethical community about likely benefits and risks that this technique could have for individuals (Macer 2005, Resnik 2017), communities and the environment, mostly due to unknown medium and long-term impacts on ecosystems.

Sterile insect technique

The SIT is based on the continuous release of large amounts of sterilised mosquito males, traditionally by means of irradiation, with the last goal to suppress vector mosquito populations. As occur with other control strategies focused on massive mosquito releases, like IIT or GMM, major challenges with SIT are related with the achievement of optimal operational costs in relation to facilities, human resources and technology need for massive mosquito rearing. Furthermore, other parameters that require an accurate scientific development like sex separation systems, adequate release methodology and exhaustive field entomological evaluations of variables like survival and sexual competition of released males, definition of suitable moments for releases according to populations phenology and monitoring the efficacy of actions conducted, among others.

Around 10 small scale field trials are projected to be held in America, Asia, Africa and Europe in next years (Bouyer *et al.* 2020), having by now some interesting preliminary results with *Ae. aegypti* and *Ae. albopictus* (Bellini *et al.* 2013, Kittayapong *et al.* 2019), both major vectors of urban arboviruses worldwide.

Wolbachia

Wolbachia is an endosymbiotic bacteria which is estimated to be naturally present in around 66% of all insect species, showing a wide range of ecological interactions, varying from parasitism, commensalism and mutualism, with their eukaryotic host cells (Dobson *et al.* 2002, Jeyaprakash and Hoy 2000). Different *Wolbachia* strains can generate parthenogenesis, feminisation and cytoplasmic incompatibility (CI) on their hosts (Jeyaprakash and Hoy 2000, Werren *et al.* 2008), being consequently an interesting biotechnological tool for insect population control. As has been described previously for GMM, there are two main approaches in the employment of *Wolbachia* to interfere on wild mosquito populations: mosquito suppression and mosquito replacement (Inácio da Silva *et al.* 2021). The first one is known as IIT and occurs when males reared in laboratory conditions which are previously infected with specific Wolbachia strains, are later released in the field to mate and reproduce with wild *Wolbachia*-free females, which finally leads to CI between gametes and the subsequent absence of viable offspring (Nazni *et al.* 2019, Yen and Barr 1971). Regarding to the second one here the goal is to replace wild populations by releasing both males and females which are infected with a specific strain of *Wolbachia* that can reduce the arbovirus replication in the target mosquito (Aliota *et al.* 2016, Dutra *et al.* 2016), so basically there is a substitution of natural populations of mosquitoes by artificial ones in order to finally reduce the vector competence of local populations. This is done thanks to the vertical transmission of *Wolbachia* from females to offspring.

Autodissemination of insecticides

One of the critical issues in the control of *Aedes* mosquitoes which usually breed in small containers of urban habitats, is the successful neutralisation of cryptic/inaccessible breeding sites in private areas. This key operational problem to control inaccessible immature mosquito habitats has been proposed in several studies to be partially solved with the employment of autodissemination strategies (Unlu *et al.* 2020). Autodissemination uses adult mosquitoes as a vehicle to treat containers that are inaccessible to direct treatments. This adult contamination can occur through direct contact of mosquitoes with treated materials (Tsunoda *et al.* 2013) or dissemination stations, such as modified ovitraps (Devine *et al.* 2009). Once adults are contaminated with the control agent, both males and females can disperse the insecticide; females usually contaminating new aquatic cryptic habitats due to oviposition actions (vertical transfer) and males also contaminating alternative females during mating thanks to polygamic behaviour (horizontal transfer). The control agents usually employed correspond to insect growth regulators (IGR's), being usually pyriproxyfen the most common insecticide. Several field studies focused on *Ae. aegypti* and *Ae. albopictus* showed promising results in terms of populations reduction (Caputo *et al.* 2012, Devine *et al.* 2009). However, there are some factors that strongly affect the degree of efficacy of autodissemination strategies which should be previously considered in each working area, such as the adult mosquito abundance, the distance between aquatic breeding sites and dissemination stations, and urban structure and topography (Seixas *et al.* 2019). Beyond *Aedes* mosquitoes and pyriproxyfen, recent researchers provided interesting results of autodissemination control assays with *Anopheles* mosquitoes and other IGR's like novaluron or triflumuron (Swale *et al.* 2018).

Attractive toxic sugar baits

This strategy is based on the sugar feeding behaviour that both males and females of mosquitoes exhibit, attracting them to bait solutions usually composed by sugar, an attractant, and an oral toxin with insecticides properties. The management of insecticide baits in case of other pest insects (cockroaches, ants, termites or non-biting flies) is conventionally widely use today. However, the development of effective and strongly attractive insecticide baits in case of hematophagic insects remains a challenge nowadays. This is one of the reasons why since the first studies conducted more than 50 years ago with promising results (Lea 1965), unfortunately this tool has never been developed and implemented in vector control programs at large scale. In this period of time, several researches have obtained interesting results in terms of mosquito control with *Aedes*, *Anopheles* and *Culex* mosquitoes, employing different substances as insecticides like boric acid, neonicotinoids, and fipronil among others (Müller and Schlein 2008, Müller *et al.* 2010a,b, Naranjo *et al.* 2013, Revay *et al.* 2014). ATSB solutions can be directly applied on vegetation or alternatively in bait stations that attract mosquitoes from a large area (the technique of lethal baits is usually named 'attract and kill'). This second type of application is suspected to have lower impact of non-target sugar feeding insects of the environment. Precisely this low knowledge of ATSB potential impact on non-target organisms, is another drawback of this strategy (Khallaayoune *et al.* 2013).

The degree of alternative sugar feeding natural sources in the control area is also another key issue to evaluate the efficacy of ATSB.

Mass trapping

Mass trapping is based on the installation of enough number of effective mosquito traps to provoke substantial reductions on mosquito populations during long terms in a specific intervention area. Depending on the target species, landscape/habitat structure (urban/rural areas, wetlands, forests, etc.) and degree of implementation of other vector control activities inside the IMMP (larviciding, adulticiding, breeding sites removal, etc.), different types of traps can be selected to achieve optimal results of mass trapping. At the end of last century, traditional ovitraps (consisting of a small black plastic container filled with water and a wooden sampling paddle to allow egg laying of mosquito females) have been modified to be lethal for ovipositing females by impregnating the oviposition substrate with insecticides (Zeichner and Perich 1999). These traps, called 'lethal ovitraps (LO)', have been deeply studied to reduce *Aedes* mosquitoes in urban areas showing positive results in trials based on massive utilisation of these devices (Ocampo *et al.* 2009, Perich *et al.* 2003, Sithiprasasna *et al.* 2003). Another modification of traditional ovitraps has derived in a tool called 'sticky ovitrap (SO)', which contains a sticky surface which also catch gravid females and resting males (Ritchie *et al.* 2003). Moreover, the last design modification of these ovitraps has allowed the development of 'autocidal ovitraps (AO)', which allow females oviposition (that is even enhanced thanks to specific attractants) but inhibit adult hatching and also allow gravid females catching (Barrera *et al.* 2014, Mackay *et al.* 2013). One of the biggest problems in the effective implementation of mass trapping strategies with ovitraps, is the negative effect of alternative and cryptic oviposition sites which are competing with LO, SO or AO. Consequently, there is a need for community engagement and participation in order to reduce alternative water-holding containers and maximise the lethal effects of ovitraps on local mosquito populations (Johnson *et al.* 2017). Beyond ovitraps, other types of conventional adult mosquito traps have been used in different studies to evaluate their significative effect on populations reduction. Various adult traps models using different attractants like ultraviolet/green-white light, carbon dioxide (CO_2), visual cues, animal baits or synthetic lures are commonly employed in the IMMP. Centers for Disease Control and Prevention (CDC) light traps, the Encephalitis-Virus-Surveillance (EVS) traps or Mosquito Magnet traps are some of these devices frequently used, but most robust scientific data about the potential use for mass trapping correspond to BG-Sentinel (BGS) traps, especially in urban areas (Akhoundi *et al.* 2018, Degener *et al.* 2014, Englbrecht *et al.* 2015, Lühken *et al.* 2014).

Complementary tools and strategies to enhance IMMP

In the current context of global change, where globalisation and climate change create new risk scenarios for the proliferation of certain vectors, the introduction of new exotic and invasive species and the consequent risk of disease transmission, it has become necessary to incorporate new technologies and integrate them into surveillance and control strategies. This allows to tackle mosquito problems from a multidisciplinary perspective, incorporating different elements that have helped to manage the problems more efficiently. In this sense, the use of geographical information systems applied to a physical and management context or the implementation of educational workshops as a source of information and awareness are clear examples of these improvements.

Geographic information systems

Geographic information systems (GIS) are nowadays widely used by professionals in mosquito research and control for computing spatially related data. There is no doubt that GIS has revolutionised the mosquito vector-management, becoming an essential tool for its monitoring and control (Bonnefoy *et al.*, 2008). Modern information technology allows the integration of GIS systems with database technology, and with digital mobile field data collection systems supported by a global positioning system (GPS). The ability to link information provides the user with a better understanding of spatial phenomena and their relationships that may not be apparent without such advanced techniques. The application of GIS allows precisely mapping locations of certain features important to the control strategy, mapping quantities and densities, e.g. over period of time in order to forecast future conditions (Becker *et al.* 2020, Khormi and Kumar 2015). Thus, GIS and information technology can greatly improve survey, logistics and documentation of mosquito control operations. The possible applications range from direct digital site-mapping using GPS assisted mobile devices to timely aggregation of operational reports. A spatially referenced database containing all features of interest is the basis for all data collection and analysis and allows for example applications as follows: Spatial analysis to determine relationships between human nuisance or disease and breeding-sites (calculation of buffer-zones, map- and database query); forecasting of time and location of appropriate control activities, based on correlations between the spatial occurrence of triggering events for larval development (e.g. water levels and flooding areas, local weather data, the potential of larval development sites, and the results of current survey data); preparation of operational maps to improve logistics, calculate the quantities of control materials and manpower required, and to calculate the duration and cost of treatment; storage of historical-site profiles and related attribute data on the basis of operational maps, enables future potential larval development, resulting from dynamic triggering events, to be predicted; GPS-assisted operations allow the tracking and direct digital documentation of field activities (e.g. aerial application). Employing a user-defined database allows precise reports and documentation of survey and control activities. The results can be visualised and printed in the form of standardised thematic maps, graphics or tables. Nowadays, knowledge of the territory and the factors favouring mosquitoes has made it possible, through spatial and geographical analysis of georeferenced data, to draw up predictive models of the risk of mosquito proliferation in a vector surveillance framework (Hay *et al.* 2006, Parra-Henao 2010). In addition, the system allows information to be added to each point on different parameters, whether structural, biological or ecological, in order to later spatially analyse this data, interpret the information in the territory and propose targeted actions which would otherwise be difficult to decide upon (Barker *et al.* 2017). Thus, identifying and register biological, social, economic, geographic and environmental data will be of great help in the development of risk maps, or predictive models that can have different uses, from the surveillance of invasive alien species and their possible routes of introduction, the selection of insecticides or the reduction of risks derived from vector-borne diseases, such as Dengue, Zika, West Nile virus or Chikungunya (Kiltron 1998).

Citizen and management science

These two elements are important in the development of IMMP in different cities of the world; the use of citizen science for the improvement municipal surveillance and control procedures, and the implementation of educative projects which collaborate with training, awareness and sensitisation through the implementation of educational workshops on vectors and their management for schoolchildren.

One example that illustrates the incorporation of citizen science is the MosquitoAlert platform, a science project to research, monitor and control the spread of disease-transmitting mosquitoes. Through the appMosquito Alert, citizens can help scientists detecting adult mosquitoes and their breeding sites (sewers, fountains, containers...) by sending pictures which are geolocated. This fact has allowed different administrations to incorporate this information into their IMMP. Moreover, recent research has revealed that this citizen science project provides a reliable and scalable tool to track disease-carrying mosquitoes (Palmer *et al.* 2017). Other citizen science project launched in 2012 named 'Mückenatlas' (mosquito atlas) show similar results in terms of its usefulness for the mosquito surveillance and control programmes (Pernat *et al.* 2021).

Moreover, it is well known that local communities are a key actor to reduce mosquito populations, since several studies have revealed that active education can lead to significant reduction in peridomestic container mosquito habitats (Bodner *et al*. 2016, Healy *et al.* 2014).

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Conclusions

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13. Integrating information on the role of mosquitoes for the transmission of pathogens of wildlife

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Abstract

Traditionally, mosquitoes have been studied given their relevance as vectors of pathogens that affect humans. However, in recent decades, their relevance as vectors of pathogens that affect wildlife has become evident. For this reason, multidisciplinary research disciplines have been developed focusing on the ecology, epidemiology and evolution of the interactions between pathogens and their hosts, including the transmission dynamics of diseases. However, there is a gap in the knowledge of mosquito-borne pathogens that affect wildlife, being necessary to study the taxa diversity, using genomic tools and, of course, their life cycles and their vectors. However, the information on the vector competence of mosquitoes for the transmission of pathogens that affect wild animals is certainly scarce. Interspecific and intraspecific differences have been evidenced. This would determine the capacity of mosquitoes to transmit parasites that infect wild animals. Different factors such as physiological and biochemical processes, or the mosquito microbiota could determine these differential capacities of mosquitoes to transmit pathogens.

Keywords: vector competence, ecology of diseases, zoonotic diseases

Introduction

This volume of the *Ecology and control of vector-borne diseases* series includes 13 chapters focused on the study of different factors affecting the role of mosquitoes in the transmission of pathogens, including those that are zoonotic. Traditionally, the study of mosquitoes derived from its role as vectors of a diversity of pathogens affecting humans. However, during recent decades, an increasing amount of evidence supports their relevance as vectors of parasites and other pathogens affecting wildlife. These studies require a multidisciplinary approach, including the collaboration between ecologists, entomologist and parasitologist, among others, to fully understand the different interactions between the three main actors: wild animals as vertebrate hosts, mosquitoes as vectors and pathogens. As a result, different research disciplines have been developed such as *disease ecology,* that focus on the study of the ecology, epidemiology and evolution of the interactions between pathogens and their hosts including the transmission dynamics of diseases.

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Mosquito-borne pathogens of wildlife

Nowadays, a diversity of mosquito-borne pathogens, including parasites, have been described in vertebrate hosts, from fishes and other ectotherms to birds and mammals. However, the lifecycle of most of them is not completely known, including basic information on the mosquito species involved in their transmission. For example, 4,654 different lineages of avian malaria and malaria-like parasites have been described in wild birds according to Malavi, the largest and updated database of these parasites (Bensch *et al.* 2009). However, information of their mosquito vectors is restricted to only 427 lineages, representing the 9.23% of known parasite lineages. In this volume, Paredes and Fuehrer (2022) and Matta *et al.* (2022) (Chapters 2 and 3, respectively) revised the published information regarding the mosquito-borne pathogens of three major vertebrate taxa, mammals, reptiles and amphibians. These authors revealed that there is a gap in the knowledge of blood parasites that affect wildlife. Thus, it is necessary to study the diversity of taxa sampled, especially using new molecular technologies such as genomic tools and, of course, to increase the knowledge on their life cycles, specifically knowing the role of the vectors. In addition to the parasites infecting wildlife in their natural ecosystems, animals maintained in captivity also provide valuable information of the pathogens able to infect these species and circulate in a given area. For example, mortality records of birds in the Bronx Zoo allowed researchers to determine the extent and timing of the WNV outbreak in 1999 in New York city, when the virus was introduced in USA (CDC 1999). The importance of zoos to facilitate transmission of pathogens to animals, has been recently supported by the local transmission of SARS-CoV-2 infections from humans to tigers and lions at the same zoo (McAloose *et al.* 2020). Mosquito-borne pathogens have also important consequences for the maintenance of animals in zoos, including endangered species. This is especially the case for parasites such as avian *Plasmodium* which are common parasites circulating in zoos in different continents and have deleterious effects on the health status of different species such as cranes and penguins, among others (Werner and Kampen 2022, Chapter 4). However, zoos also provide suitable environments to develop studies on the ecology of mosquitoes. For example, because animals are maintained in closed areas, it is possible to easily identify the flight distance of mosquitoes from their hosts to insect traps in order to estimate their flight capacity (Martínez-de la Puente *et al.* 2020), using methods to identify the blood meal origin of engorged mosquitoes (Gutiérrez-López *et al.* 2022, Chapter 6). Using this approach, it is also possible to identify their feeding preferences because the number of susceptible vertebrate hosts (enclosed animals) present in the area is known.

The role of mosquitoes in the transmission of pathogens

Vector competence is a key factor determining the ability of mosquito to transmit specific pathogens as a result of complex co-evolutionary processes (Leggett *et al.* 2013). Despite its relevance for parasite epidemiology, information on the vector competence of mosquitoes for the transmission of pathogens affecting wild animals is scarce, at least, compared to those affecting humans. Among the diversity of vector-borne pathogens infecting wild animals, only a fraction of them are able to be transmitted by mosquitoes. For example, wild birds are commonly infected by haemosporidians of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* (Valkiūnas, 2004), even coinfections by parasites belonging to these genera are common in nature (Marzal *et al.* 2008). Despite the similarities of these parasite genera in their transmission cycles, different insect groups are considered their main vectors. While *Plasmodium* is transmitted by mosquitoes, *Haemoproteus* is transmitted by louse flies and *Culicoides* and *Leucocytozoon* by black flies. As a result, when a mosquito feeds on individuals coinfected with different parasite genera, only *Plasmodium* parasites are able to complete their development (Gutiérrez-López *et al.* 2016), and only abortive forms of *Haemoproteus* could be found (Valkiūnas et al, 2013), further supporting the role of different insect groups in the transmission of phylogenetically related parasites infecting the same host species.

In addition, recent studies have provided evidence for interspecific differences in the vector competence of mosquitoes for the transmission of avian *Plasmodium*. For example, the identification of parasite DNA in the saliva of mosquitoes allowed Gutiérrez-López *et al.* (2020) to identify *Culex pipiens* as a competent vector of four parasite linages of *Plasmodium* infecting house sparrows (*Passer domesticus*) while this was not the case for mosquitoes of the species *Aedes caspius*. However, only a fraction of individuals of the same mosquito species fed on the same infected bird develop infective forms of the parasites, suggesting that intraspecific differences may also determine the ability of mosquitoes to transmit parasites infecting wild animals. Various factors may determine these differential abilities of mosquitoes to transmit pathogens, both at interspecific and intraspecific levels including physiological and biochemical processes (Abraham and Jacobs-Lorena 2004), such as the interactions of pathogens with membrane proteins of the mosquito midgut epithelium (Povelones *et al.* 2009). In addition, mosquito microbiota, through their effects on the immune responses of insects, may affect the development of parasites such as avian *Plasmodium* in mosquito vectors (Martínez-de la Puente *et al.* 2021).

Surveillance and control strategies of mosquitoes

Due to the reported importance of mosquitoes for the transmission of mosquito-borne pathogens and their impact on human and animal health, it is necessary to develop new technologies that focus on their surveillance and control of their populations. Urban areas represent suitable environments for mosquito populations, with some species being favoured by the occurrence of artificial containers and other water sources for breeding (Ferraguti *et al.* 2022, Chapter 8). This is clearly the case for *Aedes* invasive species, such as *Aedes albopictus* which has spread from the western Pacific and South-east Asia to countries in Europe, America and, Africa where it has established populations (Paupy *et al.* 2009). Among these technologies, González *et al.* 2022 (Chapter 11) highlight the role of artificial intelligence which could be an excellent tool for entomologists for the monitoring of mosquito population dynamics in the field, with the ability to detected earlier those mosquito species of interest. This would be beneficial for the design and implementation of risk assessment programs. In addition, Bueno-Mari *et al.* 2022 (Chapter 12) report the possibility of using predators, pathogens or microorganism toxins as biological control strategies to reduce mosquito populations, especially in the case of invasive species such as *Ae. albopictus*, which could be a key vector in the transmission of zoonotic pathogens from wildlife to humans.

Concluding remarks

Mosquito-borne pathogens are commonly found infecting wild animals, from ectotherms to mammals, some of them being reported as zoonotic. A growing body of evidence supports the importance of these pathogens, including mosquito-borne pathogens, as drivers of the ecology and evolution of wildlife (Rivero and Gandon 2018). Despite this, basic information on the epidemiology of these pathogens, from identification of natural reservoirs to the mosquito species involved in their transmission, is still unknown. Thus, the screening of parasite and other pathogens in wildlife is critical, especially considering that nearly 75% of emerging infectious diseases have a zoonotic origin. These studies should be complemented by those addressing the impact of environmental variables affecting the distribution and abundance of mosquitoes, considering the impact of global change potentially affecting the epidemiology of pathogens affecting wildlife (Garamszegi 2011). We hope that this volume of the *Ecology and control of vector-borne diseases* series represents a step forward in contributing to the general knowledge on this research topic.

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